50 μl of the mixture of the three mAbs at appropriate concentrations, and incubated in the dark at 4 °C for 30 min. After incubation, the cells were washed three times with 150 μl/well of FACS buffer. After the final wash, the supernatants were removed and the cells were fixed by resuspending the pellet in 200 μl of 2% formaldehyde. Flow cytometric analyses were performed using a Facscan cytometer (BD Biosciences).

2.5.2. Intracellular cytokine-staining assay

Following in vitro activation with CSFV (see above) for 28 h, the protein transport inhibitor monensin (GolgiStop™, BD Biosciences) was added to the cell cultures, and the cells were incubated for another 12 h. In some experiments, PMA (50 ng/ml) and ionomycin (500 ng/ml) were added to the culture 3 h before harvesting. Cells were harvested and stained for surface expression of CD4 and CD8 using a mixture of anti-CD4-PE and anti-CD8-PE-Cy5 conjugates (see above), resuspended in FACS buffer at the appropriate concentrations. Following the last wash, the cells were fixed and permeabilized by resuspension in 200 μl/well of Cytofix/Cytoperm solution (BD Biosciences) and incubated for 3 h, in the dark at room temperature. Intracellular IFN-γ staining was performed using biotinylated anti-swine IFN-γ mAb followed by a strepavidin-FITC conjugate resuspended in BD Perm/Wash solution provided with the BD Cytofix/Cytoperm kit (BD Bioscience). Intracellular IL-10 staining was performed using a mAb anti-swine IL-10 followed by FITC-conjugated, goat–anti-mouse IgG_1antibody. All of the washing processes following permeabilization were done using the BD Perm/Wash solution, according to the manufacturer's protocol. At the final step, the cells were resuspended in 200 μl of 2% formaldehyde and kept in the dark at 4 °C until needed. Flow cytometric analysis was performed using a Facscan cytometer (BD Biosciences).

2.6. Flow cytometric analyses

The viability of studied population prior to surface staining, determined by Trypan blue staining, was more than 80%. Numbers of gate events for analyses of surface markers and intracellular cytokines were 10,000 and 100,000, respectively. Lymphocytes were first gated (G1) from the harvested population by size (FSC) and granularity (SSC), this yielded predominantly lymphocyte population with approximately 3% $SWC3^+$ cell contamination (data not shown). The numbers of lymphocyte subpopulations ($CD4^+CD8^-$, $CD4^-CD8^+$, $CD4^-CD8^-$, and $CD4^+CD8^+$) were determined by the percentages of CD4 and/or CD8 positive cells from G1. For analyses of triple labeled cells, the G2 ($CD8^+$) and G3 ($CD4^+$) were selected from the lymphocyte (G1) population and used for determining of the expression of the second (CD4 or CD8) and third parameter (CD25 or cytokine). Since

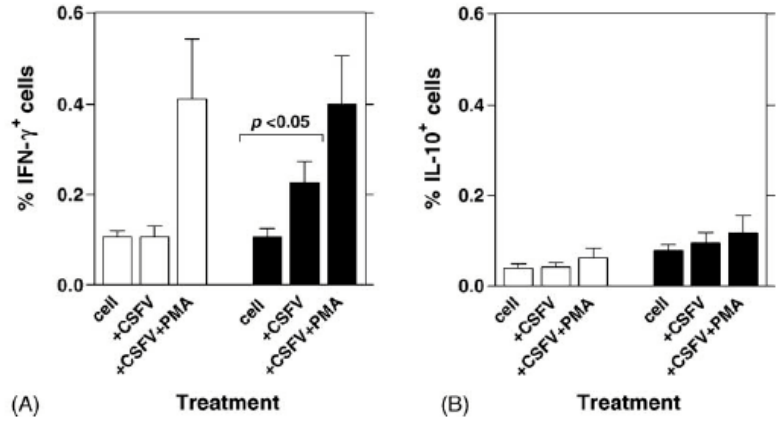

Fig. 1. Levels of IFN-γ (A) and IL-10 (B) positive cells in lymphocyte population from naïve pigs (white bar) and CSFV-primed pigs (black bar). Porcine PBMCs were in vitro cultured alone (cell), with CSFV for 40 h (+CSFV), or with CSFV for 40 h and an addition of PMA and ionomycin 3 h prior to cell harvesting (+CSFV + PMA). The data represent the mean ± S.E.M. of the percentage positive cells from five animals.

porcine $CD8^+$ population exists in $CD8^{hi}$ and $CD8^{lo}$ forms (Zuckermann, 1999), the triple positive, $CD4^+CD8^+CD25$ (or cytokine)$^+$, population were determined from G3.

2.7. Statistical analysis

All statistical analyses were performed using GraphPad Prism® Version 3.00 for Windows (GraphPad Software, San Diego, CA, USA). Either *t*-test or one-way ANOVA, followed by a post-test (Tukey's multiple comparison) when significant differences at the 0.05% confident level were present, was used as indicated.

3. Results

3.1. Interferon-γ production of by porcine lymphocytes in response to the recall antigen

Previously, porcine PBMCs have been shown to produce IFN-γ in response to the recall antigen (classical swine fever virus) by an ELISPOT assay (Suradhat et al., 2001). In this study, using the same in vitro activation system, we established an intracellular cytokine-staining assay to further characterize the CSFV-specific cytokine producing cells. The levels of cytokine production by porcine PBMCs, in response to in vitro CSFV exposure, were determined before vaccination (5 weeks old) and 2 weeks after the second immunization (9 weeks old) (see Section 2.3.1). Our result showed that the CSFV-primed lymphocytes could produce IFN-γ in response to in vitro exposure with CSFV ($p < 0.05$, *t*-test). In addition, the level of IFN-γ producing cells from the vaccinated group was significantly higher ($p < 0.05$, *t*-test) than that from the naïve group (Fig. 1A). Although lymphocytes from the naïve group did not show enhanced IFN-γ production when cultured with CSFV, the lymphocytes from both naïve and vaccinated groups produced comparable level of IFN-γ^+ cells when stimulated with PMA and ionomycin (PMA/I). This result indicated that addition of CSFV into the culture system did not affect the ability to produce IFN-γ by the lymphocytes of the naïve pigs (Fig. 1A). In the same study, addition of CSFV or CSFV and PMA/I did not significantly affect the level of IL-10 production by porcine lymphocytes from both groups (Fig. 1B).

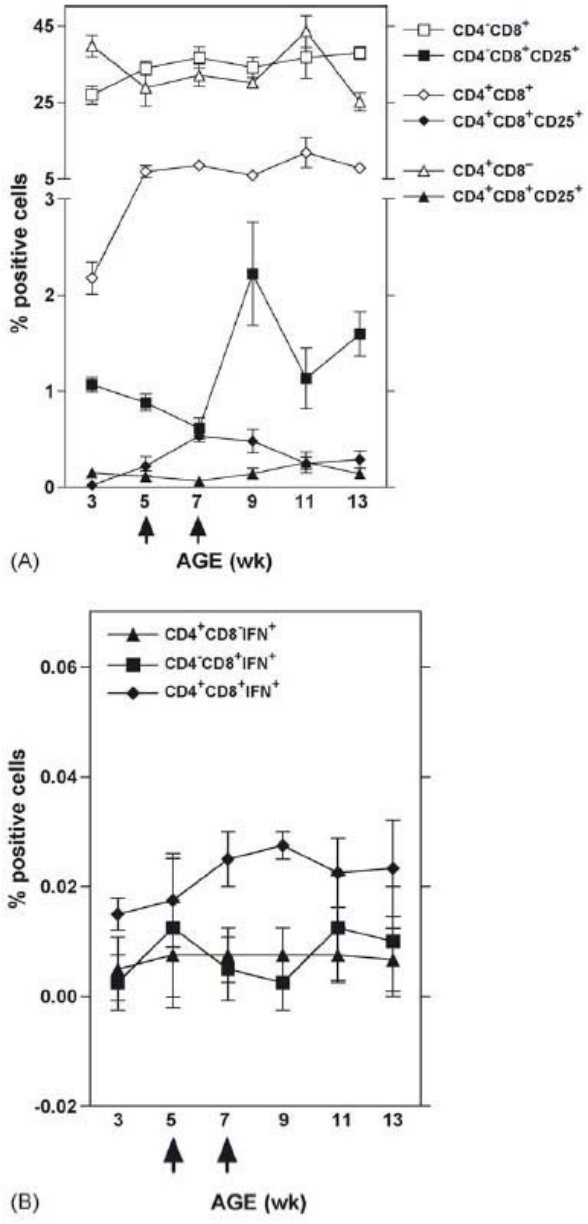

Fig. 2. Kinetics of CD25 surface expression (A), and intracellular IFN-γ production (B) in lymphocyte subsets, in PBMCs isolated from pigs vaccinated twice with CSF vaccines at 5 and 7 weeks (arrow). Porcine PBMCs were cultured in vitro with CSFV for 40 h before flow cytometric analyses. The data represent the mean ± S.E.M. of the percentage positive cells from four animals.

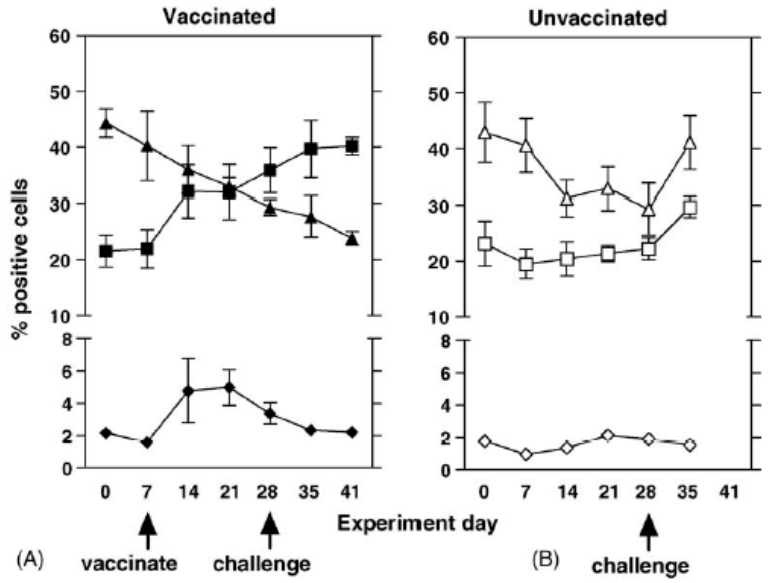

Fig. 3. Percentages of $CD4^+CD8^-$ (triangle), $CD4^-CD8^+$ (square) and $CD4^+CD8^+$ (diamond) cells from PBMCs of the vaccinated (A) and unvaccinated pigs (B). Pigs were immunized on day 7 with CSF vaccine and challenged with the virulent CSFV on at 21 dpv (day 28). Porcine PBMCs were cultured in vitro with CSFV for 40 h before flow cytometric analyses. The data represent the mean ± S.E.M. of the percentage positive cells from four animals.

3.2. Kinetics of CD25 expression and IFN-γ production by porcine PBMCs in vaccinated pigs

To investigate the relationships between CSFV-specific IFN-γ production and the expression of the IL-2R (CD25) by porcine PBMCs following vaccination, the two parameters were monitored from the same pigs at the age of 3–13 weeks old (see Section 2.3.1). Following vaccination, there was a slight increase in the activated memory lymphocyte subpopulation ($CD4^+CD8^+CD25^+$ cells), which diminished within a few weeks. The number of activated cytotoxic T cells ($CD4^-CD8^+CD25^+$ cells) peaked approximately 2 weeks after the memory population. Most of the activated lymphocyte population detected in PBMCs following in vitro activation carried the $CD4^-CD8^+$ phenotype. It should be noted that total percentage of $CD4^-CD8^+$ T cells in the PBMCs remained relatively stable throughout the experiment. Thus, it is unlikely that the increased number of $CD4^-CD8^+CD25^+$ T cells was due to a relative increase in the $CD4^-CD8^+$ T cell subpopulation (Fig. 2A). In contrast to the CD25 expression, different T cell subpopulation was found responsible for the IFN-γ production observed in the PBMCs following CSFV vaccination. The result showed that $CD4^+CD8^+$ T cells were the major IFN-γ producers in PBMCs throughout the experiment, while the number of IFN-γ

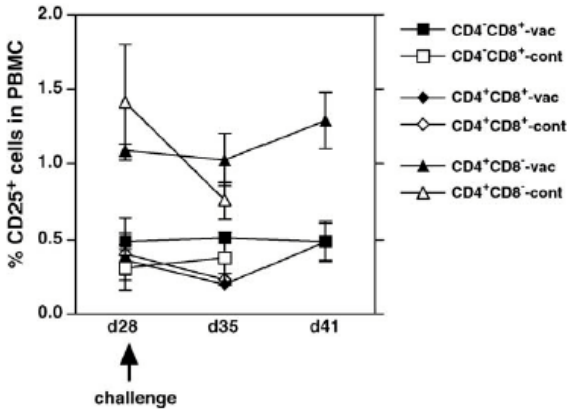

Fig. 4. Kinetics of CD25 surface expression on lymphocyte subsets in the PBMCs of pigs vaccinated with CSF vaccines and challenged with virulent CSFV at 21 dpv (day 28). Porcine PBMCs were cultured in vitro with CSFV for 40 h before flow cytometric analyses. The data represent the mean ± S.E.M. of the percentage positive cells from four animals.

producing $CD4^{-}CD8^{+}$ cells was relatively low and comparable to that of the IFN-γ producing $CD4^{+}CD8^{-}$ cells (Fig. 2B). In addition, the kinetic of IFN-γ production was resembled to that of activated $CD4^{+}CD8^{+}$ T cells. In this experiment, cellular activation and IFN-γ production in the naive $CD4^{+}CD8^{-}$ population following vaccination was minimal (Fig. 2).

3.3. Kinetics of CD25 expression and cytokine production by porcine PBMCs following CSFV challenge

To determine the kinetics of CD25 expression and cytokine production following the CSFV challenge, crossbred pigs (4 pigs/group) were immunized once with a CSF vaccine and challenged with a virulent

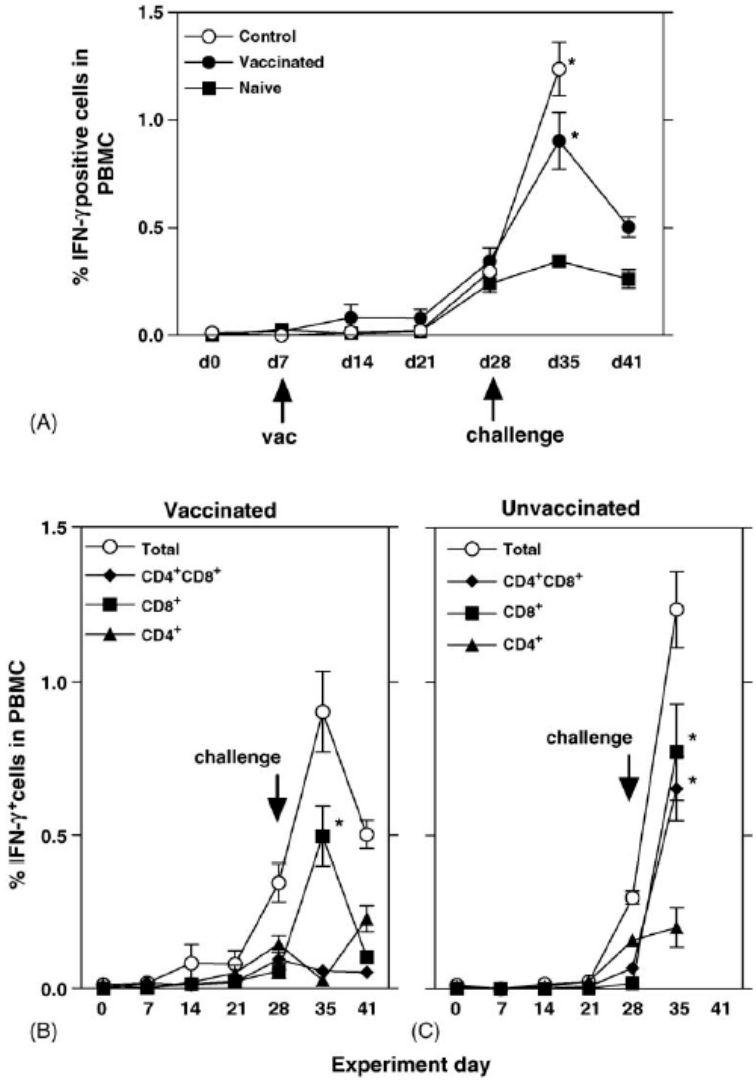

Fig. 5. Kinetics of total IFN-γ production by porcine lymphocytes (A) and by lymphocyte subsets from the PBMCs of vaccinated (B) and unvaccinated (C) group. Pigs were vaccinated with CSF vaccines (day 7) and challenged with virulent CSFV at 21 dpv (day 28). Porcine PBMCs were cultured in vitro with CSFV for 40 h before flow cytometric analyses. The studied lymphocyte subsets included $CD4^{+}CD8^{-}$ ($CD4^{+}$), $CD4^{-}CD8^{+}$ ($CD8^{+}$) and $CD4^{+}CD8^{+}$ populations. The data represent the mean ± S.E.M. of the percentage positive cells from four animals. The symbol (*) indicates significant difference from the data of day 28 ($p < 0.05$, paired t-test).

strain of CSFV (Bangkok 1950) at 21 dpv (Section 2.3.2). Unvaccinated pigs also received the same amount of the challenge virus at the same time. Following the CSFV challenge, the unvaccinated pigs exhibited severe clinical signs of CSF starting from 7 days post-infection (dpi). All of the unvaccinated pigs developed severe leukopenia, with insufficient cells to analyze after the first week of infection, and died within 14 days following the challenge. Therefore, there was no data on the lymphocyte subpopulation from the control pigs after day 35. All of the vaccinated pigs and naïve pigs remained clinically normal through the end of the experiment.

Following vaccination, the numbers of $CD4^{-}CD8^{+}$ and $CD4^{+}CD8^{+}$ from vaccinated group slightly increased, compared to those from the control group (Fig. 3). However, due to variation of the lymphocyte numbers from pigs within the same group, the changes in the lymphocyte numbers between days 7 and 14 were not statistically significant. Nevertheless, the number of $CD4^{-}CD8^{+}$ cells of the vaccinated group gradually increased, while the number of $CD4^{+}CD8^{-}$

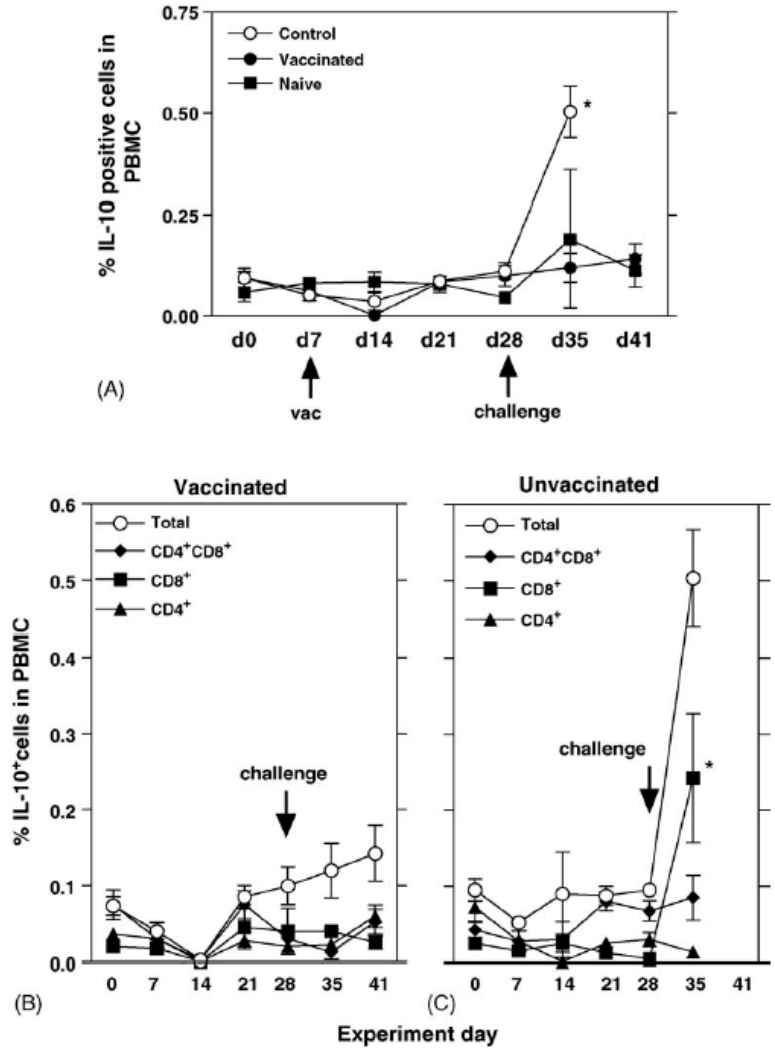

Fig. 6. Kinetics of total IL-10 production by porcine lymphocytes (A) and by lymphocyte subsets from the PBMCs of vaccinated (B) and unvaccinated (C) group (see Fig. 5). The symbol (*) in part (A) indicates significant difference from other groups ($p < 0.05$, ANOVA followed by Tukey's multiple comparison test). The symbol (*) in part (C) indicates significant difference from the data of day 28 ($p < 0.05$, paired t-test).

cells decreased, after vaccination through the end of the experiment (Fig. 3A). Numbers of the studied lymphocyte subpopulation of the control group remained relatively unchanged throughout the experiment (Fig. 3B).

At the time of challenge (day 28), no significant differences in the numbers of $CD25^+$ and IFN-γ^+ cells among the groups were observed (Figs. 4 and 5). Interestingly, the numbers of $CD25^+$ cells among the studied populations also remained unchanged following the challenge (Fig. 4). In contrast to the pattern of CD25 expression, there were significant increases in the numbers of IFN-γ producing cells in both vaccinated and unvaccinated groups following challenge (Fig. 5A). Exposure to virulent CSFV significantly increased the number of IFN-γ producing cells in the PBMCs of both vaccinated ($p < 0.05$, paired *t*-test) and unvaccinated pigs ($p < 0.01$, paired *t*-test) at 7 dpi. The numbers of IFN-γ producing cells in the vaccinated group fluctuated after challenge. At day 35, the $CD4^-CD8^+$ population was major IFN-γ producing cells in vaccinated pigs, while both $CD4^+CD8^+$ and $CD4^-CD8^+$ populations contributed to the IFN-γ production in the unvaccinated group (Fig. 5B and C). Although, the number of IFN-γ producing cells in the unvaccinated group markedly increased by 7 dpi, these pigs did not survive the challenge. Interestingly, the number of IL-10 producing cells in the unvaccinated group also significantly increased and higher than the other groups at 7 dpi ($p < 0.05$, ANOVA followed by Tukey's multiple comparison test) (Fig. 6A). The majority of the IL-10 producing cells were the $CD4^-CD8^+$ population (Fig. 6C). No significant changes in IL-10 production were observed in the vaccinated group following the challenge (Fig. 6A).

4. Discussion

Like other species, pigs have typical $CD4^+CD8^-$ and $CD4^-CD8^+$ T lymphocytes in their peripheral blood and lymphoid tissues. These cells have been shown to have helper and cytolytic functions, respectively. However, unlike humans and mice, pigs also have an extrathymic double positive lymphocyte population that comprises 8–64% of the circulating pool of small resting T cells (reviewed in Zuckermann, 1999). It was recently shown that the extrathymic naïve Th cells ($CD4^+CD8^-$) can upregulate their CD8 expression upon cellular activation. Therefore, by examining the expression of the CD4 and CD8 molecules, porcine Th populations can be categorized into resting Th cells ($CD4^+CD8^-CD25^-$), activated Th cells ($CD4^+CD8^-CD25^+$) and memory (or primed) Th cells ($CD4^+CD8^+CD25^-$) (Saalmuller et al., 2002). In this experiment, we followed the number of single positive and double positive porcine lymphocyte subsets in PBMCs following exposure to CSF antigen. We also monitored the expression of the activation marker (CD25) and intracellular IFN-γ production in these subpopulations. It should be noted that the PBMCs were cultured in vitro with CSFV before flow cytometric analyses. Therefore, kinetics of cellular activation and cytokine production obtained from this experiment would reflect CSFV-specific responses in the PBMCs.

It has been shown in several studies that the level of IFN-γ production can be used as an indicator for cell-mediated immunity in pigs (Mateu de Antonio et al., 1998; Zuckermann et al., 1998; Suradhat et al., 2001). Our result from this study clearly showed that primed porcine PBMCs produced IFN-γ, but not IL-10, in response to secondary exposure to CSFV, and that the response could be measured by flow cytometry (Fig. 1). Following vaccination with CSF vaccine, double-positive (DP), $CD4^+CD8^+$ cells were the major IFN-γ producers in response to in vitro activation (Fig. 2). This finding is in agreement with previous reports showing that the DP cells are a memory population, which can produce high levels of IFN-γ in response to a recall antigen or polyclonal activator (Rodriguez-Carreno et al., 2002; Saalmuller et al., 2002). The finding also implies that that the IFN-γ producing cells detected by ELISPOT assays following immunization with a CSF vaccine (Suradhat et al., 2001; Suradhat and Damrongwatanapokin, 2003) were indeed reflecting helper T lymphocyte (Th) activity. It should also be noted that the number of IFN-γ producing cells following vaccination was not as high as that observed following the viral challenge. This finding is consistent with a previous report showing that immunization with a modified live vaccine induces a lower level of cell-mediated response than infection (Piriou et al., 2003). In Experiment 2, the vaccinated group did not show any

significant increase in the number of IFN-γ producing cells following vaccination, compared to a sharp increase in the numbers of IFN-γ producing cells following the CSFV challenge (Fig. 5). The indifferences in cytokine production among the groups following vaccination might be partly related to the low levels of detectable IFN-γ producing cells, and the high variation of the numbers of cytokine producing cells from pigs within the same group. In our experience, detection of IFN-γ producing cells following CSF vaccination by flow cytometry is not always as sensitive as the previously reported ELISPOT assay. It should be noted that on day 28 of Experiment 2, the numbers of IFN-γ producing cells in all groups increased considerably without any specific treatment (Fig. 5A). The reason for this fluctuation is no clearly known, but could relate to non-specific activation of the lymphocytes, as there seemed to be increased activity on the cytokine production of the naïve $CD4^+$ cell (Fig. 5B and C).

Following vaccination for CSF, there seemed to be two phases of cellular activation measured by the upregulation of surface CD25 expression; early cellular activation of the DP population is followed by the activation of the $CD4^-CD8^+$ T lymphocytes (Fig. 2A). Although the CD25 marker has been used to identify cellular activation in several reports (Dillender and Lunney, 1993; Quade and Roth, 1999; Saalmuller et al., 2002; Piriou et al., 2003), however, we did not see a good correlation between the level of CD25 expression and cytokine production in PBMCs either following vaccination or after challenge (Figs. 2 and 4). In this study, the activated population might not always be the major population producing the cytokine in response to the CSFV antigen. The differences in dynamics of CD25 expression and cytokine production might be related to the specific lymphocyte population in each study. Unlike the cytokine production, which is shown to be antigen-specific (Fig. 1), combining the percentages of $CD25^+$ cells from the three studied lymphocyte subpopulations ($CD4^+$, $CD8^+$, and double positive) contributed to less than 50% of the $CD25^+$ cells from total lymphocyte population, in both naïve and primed populations (data not shown). In addition, no significant differences in the numbers of $CD25^+$ cells were observed in the challenge pigs regardless of their immunological history (Fig. 4). These results suggested that lymphocytes could also be activated, non-specifically, and upregulated CD25 expression by CSFV. Alternatively, it is also possible that the incubation period for the in vitro activation system used in this study is shorter than in previous reports (Saalmuller et al., 2002; Piriou et al., 2003). Therefore, the results could reflect the different dynamics of cellular activation at a different incubation time. Development of the four-color staining assay that simultaneously analyses CD25 expression and IFN-γ production, in addition to the surface markers, will provide a better explanation on this discrepancy.

Following challenge with CSFV, the number of IFN-γ producing cells in the PBMC population increased in both vaccinated and unvaccinated pigs at 7 dpi (Fig. 5). Interestingly, the $CD4^-CD8^+$ population was the subpopulation mainly responsible for IFN-γ production in the PBMCs of both groups following challenge, while there were a significantly less number of IFN-γ producing DP cells in the PBMCs of the vaccinated group during the first week of infection (Fig. 5A). This finding is in agreement with the previous report demonstrating that the activation of $CD8^+$ CTLs was observed early (from 15 dpi) in the PBMCs of immunized pigs challenged with CSFV, but the activation of $CD4^+CD8^+$ cells was not observed until 35 dpi (Piriou et al., 2003). As it has been previously shown that the porcine memory population preferentially home to the secondary lymphoid organs (Zuckermann, 1999). The low number of antigen-specific DP cells detected in the PBMCs of primed pigs following the CSFV challenge could simply reflect the difference in tissue homing preference among the subpopulations during an effector phase of the immune response.

It should be noted that porcine gamma–delta (γδ) T lymphocytes can also expressed the CD8 surface molecules (Pescovitz, 1998). However, the proportion of γδ T lymphocytes in the PBMCs of young pigs is usually low and they are not the major IFN-γ producers in the PBMCs even when stimulated with a polyclonal T cell activator (Rodriguez-Carreno et al., 2002). In addition, γδ T cells are more sensitive to CSFV infection and are depleted early after the viral challenge (Pauly et al., 1998). Although the kinetics of CD25 expression and IFN-γ production by γδ T lymphocytes were not monitored in this study, it is unlikely that the γδ T cells be the major IFN-γ

producers observed in the $CD4^{-}CD8^{+}$ population following the viral challenge.

Although the number of IFN-γ positive cells was markedly increased in the unvaccinated pigs at 7 dpi (Figs. 3 and 4), these pigs did not survive the CSFV challenge. This phenomenon, which is usually seen in pigs with overwhelming CSFV infections, has been previously demonstrated using an ELISPOT assay (Suradhat et al., 2001). Interestingly, the number of IL-10 producing cells in the PBMCs was also markedly increased in the unvaccinated pigs, while the level of IL-10 production in the vaccinated pigs remained low through the end of the experiment (Fig. 6). The $CD8^{+}$ cells were also found the major IL-10 producer in PBMCs (Fig. 6C). Thus, our results demonstrated that CSFV infection significantly increased the levels of both IFN-γ and IL-10 production in unvaccinated pigs. It should be noted that although CSFV is not usually detected in the PBMC population during the first week of infection, but abrogation of cellular immune responses is observed as early as 5 dpi (Pauly et al., 1998). Interleukin-10 is widely accepted to be a potent immunosuppressive cytokine that can strongly inhibit both innate and specific immune functions (Moore et al., 2001; Redpath et al., 2001). Our findings suggest that cytokine dysregulation could be one of the underlying mechanisms resulting in the immunoinhibitory effect observed following CSFV infection. Recently, the immunoinhibitory effects of viral infection via IL-10 induction have also been observed in hepatitis C virus (Dolganiuc et al., 2003) and human immunodeficiency virus (Almonti et al., 2003) models.

Taken together, our results suggest that CD25 expression and antigen-specific IFN-γ production by porcine lymphocytes may not be tightly associated. Furthermore, different lymphocyte populations appear to be responsible for cytokine production in the PBMCs following CSF vaccination and challenge. Further understanding of the kinetics of the cellular response against CSFV may provide information about the mechanisms of protection and a better strategy for disease control in the future.

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IMPACT OF PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS INFECTION ON THE EFFICACY OF CLASSICAL SWINE FEVER VACCINE

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Running title: Impact of PRRSV on CSF vaccination.

Abstract

Recent findings suggest that porcine reproductive and respiratory syndrome virus (PRRSV) possesses immunomodulatory properties. To investigate the effect of PRRSV infection on classical swine fever (CSF) vaccine efficacy, twenty-eight, 17-day-old pigs were divided into 5 groups. The experimental group was infected with a Thai PRRSV (01NP1) a week before CSF vaccination and challenged with the virulent CSF virus (Bangkok 1950) 3 weeks later. The control groups received no PRRSV infection, no CSF vaccination, no CSF challenge or in combination were included. The results demonstrated that PRRSV infection significantly reduced the CSF vaccine efficacy and could cause vaccination failure in the field.

Keywords: PRRSV, CSFV, vaccine

1. Introduction

Classical swine fever (CSF) is one of the most important viral infectious diseases of domestic pigs, causing substantial economic losses to the swine industry in most part of the world [1]. Classical swine fever is caused by an enveloped, single-stranded RNA virus, belongs to the pestivirus genus, of the Flaviviridae family [2]. The clinical outcome of CSFV infection is varied, from acute, subacute to chronic forms, depending on the virulence of the virus variants [3]. In endemic areas, vaccination against the CSFV is the most common means for disease control. The CSF vaccine derived from the Chinese (C)-strain, is considered to be safe and very effective in the induction of protective immunity against CSF. The vaccine has been shown to induce complete protection against CSFV infection [4], and has been extensively used for the control of CSF in domestic pigs, in particular in the highly endemic areas. Generally, the C-strain CSF vaccine induces complete clinical protection against either homotypic or heterotypic CSFV strains within the first week after vaccination [5-8]. Although, the C-strain derived CSF vaccines can efficiently induce protective immunity against CSFV infection, several other factors can influence the efficacy of the vaccine. These factors include the level of maternal derived antibodies at the time of vaccination [4, 8-10], vaccination protocol [5], age of the pig and co-infection with other pathogens [11].

Porcine reproductive and respiratory syndrome virus (PRRSV), an enveloped positive-stranded RNA virus, is a member of the family Arteriviridae in the order Nidovirales [12]. Since its emergence in the late 1980's, PRRSV has been recognized worldwide as one of the most economically important pathogens of swine [13]. The major characteristics of porcine reproductive and respiratory syndrome (PRRS) include reproductive failure of the sows and respiratory disease in pigs of all ages. Several studies suggest that PRRSV may negatively modulate the host immune responses. PRRSV infected pigs usually demonstrate prolonged viremia and persistent infection [14]. In addition, weak innate immunity followed by delayed and inefficient specific immunity is usually observed following PRRSV infection [14-16]. Infection with PRRSV can also result in declined local lung defenses leading to secondary bacterial infections which are known as the porcine respiratory disease complex [17]. One of the postulate mechanisms for immunomodulation is an induction of a potent immunosuppressive cytokine, interleukin (IL)-10, both systemically and locally, during an early stage of PRRSV infection [18-21]. Inhibition of the immune cells by IL-10 could be one of the explanations for the suppressed lung defense observed following PRRSV infection, as cell-mediated immunity is believed to play a significant role in respiratory defense mechanisms [22].

Apart from the respiratory defense, several lines of evidence suggest that the immunomodulatory effect of PRRSV may confer a negative impact on the immune responses induced by other vaccines. PRRSV infected pigs exhibit altered magnitude of T cell responses to pseudorabies vaccine [23]. Infection or vaccination with PRRSV appears to decrease the efficacy of M. hyopneumoniae bacterin in M. hyopneumoniae challenged pigs [24]. Recently, it has been shown that PRRSV infection suppresses the antibody response to CSF vaccine [25] and significantly reduced the CSFV-specific interferon (IFN)-γ production by the CSF vaccine-

primed peripheral blood mononuclear cells (PBMC) [26]. These findings suggested that PRRSV infection could affect both cell-mediated and humoral immunities induced by the CSF vaccine. However, there is still limited information of PRRSV infection on the efficacy of CSF vaccine against the field challenge. In this study, we investigated the effect of PRRSV infection on the efficacy of CSF vaccine, using a previously established experimental challenge model. In order to understand the mechanism of immunosuppression, the systemic productions of the cytokines, IFN-γ and IL-10, by porcine lymphocytes were monitored by flow cytometry.

2. Materials and methods

2.1 Cells and viruses

The CSFV reference strain, ALD strain, was a gift from the National Institute of Animal Health of Japan. The CSFV strain used for challenges was the Thai isolate (Bangkok 1950 strain) from the National Institute of Animal Health of Thailand (NIAH). Viruses were propagated in a SK-6 cell line. Infected cells were collected after 4 days post infection, and subjected to 2 freeze-thaw cycles. The viral suspension was centrifuged at 1000 g for 20 minutes. The stock viruses in supernatant were stored at -80o C until needed. Viral titers were determined by a peroxidase-linked virus titration assay as described previously [27].

Thai PRRSV field isolate (strain 01NP1) was recovered from the pooled sera of PRRSV-infected pigs and designated as the North American genotype [28]. The virus was cultured in MARC-145 cells and stored at -80 oC until needed. Virus isolation and titration were performed in Marc-145 cell line as previously described [29].

2.2 Animals and experimental protocols

Twenty-eight, 17-day-old, crossbred pigs from a CSF-free, PRRS-free commercial farm were housed at the animal facility, Faculty of Veterinary Science. The experimental and animal handling protocols were approved by the Ethics Committee on Experimental Animal Usage and Animal Welfare, Faculty of Veterinary Science, Chulalongkorn University.

The pigs were sero-negative to PRRSV, but possessed anti-CSFV maternal derived antibody titers (MDA), due to a routine CSF vaccination program in the sows. Five to seven pigs were randomly grouped into 5 experiment groups (table 1). On day 0 (D0), group C and D were intranasally inoculated with 5 ml (2.5 ml/nostril) of the Thai isolate PRRSV (strain 01NP1), at the titer of 104.5 TCID50/ml. The other groups (A, B and E) were mock infected with an equal volume of MARC-145 lysate. On day 7 (D7), group B and D were immunized intramuscularly with a modified live, lapinized Chinese strain, CSF vaccine (1ml/dose), (Department of Livestock Development, Bangkok, Thailand). Other groups received an equal amount of vaccine diluent. The means maternal derived serum-neutralizing antibody titers of all groups were less than 8 at the time of vaccination. At 21 days post vaccination (D28), the pigs (group A-D) were challenged intramuscularly with 2x104 TCID50 of the virulent CSFV, strain Bangkok 1950 (NIAH, Thailand). The negative control pigs (group E) were kept in a separate isolation unit throughout the experiment. Clinical signs and rectal temperatures were monitored daily. Total leukocyte count

was performed at day 0, 3, 5, 7 and 14 post challenge. Blood and serum samples were collected for CMI assay, virus isolations, and neutralizing antibody titration every week until 2 weeks after the challenge (D41). On D41, all survived pigs were euthanized and necropsied for examination of pathological changes and virus isolation.

2.3 Detection of viral specific antibody titers

Serum neutralizing (SN) antibody titers against CSFV were determined by neutralizing peroxidase linked assay (NPLA) as described previously [5]. SN titer was the reciprocal of the highest dilution of the serum that completely inhibited viral infection.

Total anti-PRRSV antibody titers were determined by a commercial ELISA (HerdChek PRRSV IDEXX Laboratories, Westbrook, ME) according to the manufacturer's protocol. The nominal cut-off for a positive result was a sample/positive (S/P) ratio of 0.4.

2.4 Isolation of porcine PBMCs

Porcine peripheral blood mononuclear cells (PBMCs) were isolated from 10 ml of the heparinized blood samples using Isoprep® separation medium (Robbins Scientific Cooperation, Sunnyvale, CA) according to the manufacturer's protocol. The purified PBMCs were resuspended at a concentration of 6x106 cells/ml in RPMI 1640 (GIBCO, Carlsbad, CA), supplemented with 10% calf serum (Starrate, Bethungra, NSW, Australia), 2mM L-glutamine (GIBCO), 100 μM non-essential amino-acid (GIBCO), 1 mM sodium pyruvate (GIBCO), 50 μM 2-mercaptoethanol (Sigma Chemical Co., St. Louis, MO) and 100 unit/ml of penicillin G, 100 μg/ml of streptomycin and 0.25 mg/ml of amphotericin B (antibiotic/antimycotic solution; GIBCO).

2.5 Antibodies and reagents

The custom PE-Cy5 conjugated anti-swine CD8 monoclonal antibody (mAb) (76-2-11, IgG2a) was kindly provided by Dr. James A. Roth (Iowa State University, Ames, Iowa, USA). Anti-swine CD4-FITC conjugated mAb (74-12-4, IgG2b), anti-CD4-PE conjugated mAb (74-12-4, IgG2b), and biotinylated anti-swine IFN-γ mAb (P2C11, IgG2a) were purchased from BD Biosciences (San Diego, CA). The mAb anti-swine IL-10 (945A4C437B1, IgG1) was from Biosource International Inc (Nivelles, Belgium). Streptavidin-FITC and goat-anti-mouse IgG1-FITC conjugates were from Serotec (Oxford, UK).

2.6 Flow cytometry

2.6.1 In vitro activation and surface staining

One ml of freshly isolated PBMCs (6x106 cells/ml) was cultured in a well of 24-well plate, with CSFV (ALD strain) at 1 multiplicity of infection (m.o.i.). At 28 hours post inoculation, the protein transport inhibitor monensin (GolgiStop™, BD Biosciences) was added to the cell cultures, and the cells were incubated for another 12 hr. The cells were harvested for surface marker staining at 40 hours post inoculation. Briefly, approximately 2x106 cells were distributed into a well of round-bottom tissue culture treated 96-well-plate, washed with PBS supplemented

with 0.5% bovine serum albumin (Sigma) and 0.1% sodium azide (Sigma) (FACS buffer). Duplicate plates were set identically, one set for intracellular IFN staining and another for intracellular IL-10 staining. The cells were pelleted by centrifugation at 500 g for 2 min. The supernatants were discarded and the pellets were stained with 50 μl of the mixture of the mAbs (anti-CD4-FITC, anti-CD8-PE-Cy5) at their previously titrated optimum dilutions, and incubated in the dark at 4oC for 30 min. The cells were then washed 3 times with 150 μl/well of FACS buffer.

2.6.2 Intracellular cytokine staining

Following the last wash, the cells were fixed and permeabilized in 200 μl/well of Cytofix/Cytoperm solution (BD Biosciences) and incubated for 3 hr, in the dark at room temperature. The cells were washed 3 times with 150 μl/well BD Perm/Wash solution, according to the manufacturer's protocol. Fifty l of biotinylated mouse anti-swine IFN-γ diluted in BD Perm/Wash solution was added to all wells and incubated in the dark at 4oC for 30 min. After 3 washes, 50 l of streptavidin-FITC conjugate was added to all wells and incubated in the dark at 4oC for 30 min. All of the washing processes following permeabilization were done using the BD Perm/Wash solution, according to the manufacturer's protocol. At the final step of the 3 washes, the cells were resuspended in 200 μl of 2% formaldehyde, transferred to a centrifuge tube and kept in the dark at 4oC until analyzed. For intracellular IL-10 staining the same method was applied but the primary antibody was mouse anti-swine IL-10 mAb and the secondary antibody was FITC-conjugated, goat-anti-mouse IgG1 antibody. Flow cytometric analysis was performed using a Facscan cytometer (BD Biosciences).

2.6.3 Flow cytometric analyses

The viability of studied population prior to surface staining, determined by Trypan blue staining, was more than 80%. Numbers of events collected for analyses was 100,000. Lymphocytes were first gated (G1) from the harvested population by size (FSC) and granularity (SSC), this yielded predominantly lymphocyte population with approximately 3% SWC3+ cell contamination (data not shown). For analyses of triple labeled cells, the G2 (CD8+) and G3 (CD4+) were selected from the total lymphocyte population (G1) and used for determining of the expression of the second (CD4 or CD8). Since porcine CD8+ population exists in CD8hi and CD8lo forms [30], the triple positive, CD4+CD8+cytokine+, population was determined from G3.

2.7 Statistical analysis

All statistical analyses were performed using GraphPad Prism® version 3.00 for Windows (GraphPad Software Incorporated, San Diego, CA).

3. Results

3.1 Clinical signs and survival rates of the experimental pigs

The detail of the group treatments and number of experimental pigs were shown in Table 1. Following PRRSV infection on day 0 (D0), the pigs (group C and D) developed clinical signs of PRRS during day 2-16. The observed clinical signs included depression, anorexia, conjunctivitis, puffy eyes, and respiratory distress. The respiratory sign was most prominent during day 4-5 and recovered by day 16 of the experiment. Three pigs from group C and one pig

from group D died, from Haemophilus spp. infection during day 10-18 post PRRSV infection. The rest of the pigs receiving MARC-145 mocked infected cell lysate (group A, B, and E) remained healthy up to the time of CSFV challenge. No adverse effect was observed following vaccination with CSF vaccine on day 7. The negative control pigs (group E) remained clinically normal until the end of the experiment.

Following the CSFV challenge, the control-vaccinated pigs (group B) remained clinically normal, and did not show significant changes in leukocyte count (figure 1), suggesting that the C-strain vaccine induced complete protection against the CSFV challenge. In contrast, the unvaccinated pigs (group A and C) exhibited a severe clinical picture of CSF including high fever (rectal temperature >40oC), depression, anorexia, leukopenia, respiratory distress, tremor, diarrhoea, petechial haemorrhage of the skin, and nervous signs in the late stage of infection. The challenged pigs were dead or euthanized, due to severe clinical signs, between days 37-39 (group A) and days 33-37 (group C). None of the unvaccinated pigs (group A and C) survived the challenge. The vaccinated pigs that were previously infected with PRRSV (group D) exhibited clinical signs of CSF infection, with a milder degree comparing to the unvaccinated groups. However, the pigs were severe leukopenic comparable to group A and C. Most of the pigs in this group died between days 33-36. At the end of the experiment, there was only one pig from group D survived with evidence of recovering leukocyte number (figure 1). The survival rate of the group D was significantly different ($p<0.05$, Fisher's exact test) from group B and was statistically indifferent from group A and C. Bacteriological studies revealed significant bacterial complication in the groups A, C and D. The identified bacteria included Streptococcus spp., Haemophilus spp., and E. coli (data not shown).

3.2 Virological studies and pathological findings

Following PRRSV infection, PRRSV could be detected in the sera of infected pigs from day 7 and disappeared by day 28 in the non-vaccinated group (group C). Interestingly, in the CSF-vaccinated group (group D), PRRSV could be isolated from the serum samples up to the time of CSFV challenge and persisted until the time of death (table 2).

Classical swine fever virus was detected in the sera from the non-vaccinated groups (A and C) from day 31 and markedly increased until the time of death. No CSFV was detected in the sera of the vaccinated pigs (group B), indicating that these pigs were completely protected by CSF vaccination. In contrast, the vaccinated pigs that were previously infected with PRRSV (group D) exhibited considerably high amounts of CSFV in the sera following the challenge. The reduced CSFV titer detected on day 38 was from the only one survived pig in this group (Table 2). Consistent with the results from sera, significantly high CSFV titers were found in the internal organs of the unprotected pigs (group A, C and D). The PRRSV-infected, vaccinated group (group D) contained significantly higher CSFV titers in the internal organs compared to the vaccinated counterpart (group B). In most of the studied organs, the CSFV titers from the group D were comparable to those from the non-vaccinated control pigs (group A). The PRRSV-infected, non-vaccinated pigs (group C) contained higher amounts of CSFV than the CSFV

challenge group (group A) especially in kidney and lymph nodes. No CSFV was isolated from the pigs from group B and E (table 3). The pathological changes and percentages of pigs exhibited the macroscopic lesions were described in table 4. The findings were consistent with the clinical and virological findings.

3.3 Immunological responses and cytokine productions

The serological study on the level of anti-PRRSV antibodies revealed that the most of the pigs seroconverted (S/P ratio > 0.4) by 2 weeks post infection. It should be noted that the antibody response of the vaccinated group (group D) tended to develop slower than the non-vaccinated group (group C), and that the antibody titers still increased up to the end of the experiment (table 5).

Since the experimental pigs were obtained from a commercial farm, in which CSF vaccination was routinely practiced, the pigs therefore, possessed certain levels of maternal-derived antibody (MDA) titers. On day 7 of the experiment, all of the experimental groups had comparable level of mean MDA titers ($p > 0.05$, ANOVA). The mean titer of every group was less than 32 which has been previously demonstrated to have minimal interfering effect on the induction of anti-CSFV immunity by vaccination [8, 10]. Following vaccination, the vaccinated pigs (group B) seroconverted from 7 days post vaccination. Furthermore, the pigs exhibited enhanced antibody responses following the CSFV challenge, suggesting the induction of anamnestic responses. No significant enhancement in anti-CSFV antibody responses was observed in the PRRSV infected, vaccinated pigs (group D), even after the CSFV challenge (figure 2). At the time of challenge (day 28), the mean anti-CSFV neutralizing titers of group D was significantly lower than that of the group B ($p < 0.05$, student t-test).

The study of CSFV-specific IFN-γ production by flow cytometry revealed no enhancement of IFN-γ production following vaccination in any group. However, following CSFV challenge, significant increases of IFN-γ+ cells were observed in all the challenged groups (figure 3) with the evidence of the CD8+ cells as a major IFN-γ producer in every group (data not shown). The vaccinated pigs exhibited a significant enhanced IFN-γ production ($p < 0.05$, paired t-test), which was then reduced to a background level, compared to the control pigs, suggesting that the priming effect and controlled infection occurred in this group (figure 3). The non-vaccinated pigs (group A and C), and the PRRSV-infected, vaccinated pigs (group D) exhibited sharp increases of IFN-γ production following the CSFV challenge. By day 35, the animals in the non-vaccinated, challenged groups (A and C) started to die or were severely leukopenic (see figure 1) with insufficient cell numbers to be analyzed statistically. The data of group D on day 41 was obtained from the only survived pig, which showed recovering signs, with the level of IFN-γ producing cells declined to the background level (figure 3).

Interestingly, when analyzing the same set of cultured cells, there were increasing number of IL-10+ cells in the PRRSV infected group which peaked on day 14, and also following the CSFV challenge. The non-vaccinated, challenged pigs (group A) also exhibited increased IL-10 production following the challenge (figure 3). The CD8+ was the major subpopulation in IL-10

production (figure 4). Due to the high variation of the number of IL-10 producing cells within the group, no statistically difference could be analyzed. Nevertheless, these results demonstrated more fluctuation in IL-10 production in the groups that were previously infected with PRRSV.

4. Discussion

In the endemic area, routine vaccination is one of the most effective strategies for prevention and control of CSF. The modified live, C-strain, CSF vaccine is regarded as one of the most effective CSF vaccines that provides not only clinical but also complete virological protection, i.e. sterile immunity, within a week following vaccination [4, 5]. The C-strain vaccine has been shown to induce heterotypic protection against all the subtypes circulated in Thailand including the recently reported variants found only in Asia, the genogroup 3 [6, 7, 27]. In this study, a complete protection including prevention of leukopenia and viremia, induced by CSF vaccination was again confirmed (group B). However, PRRSV infection prior to CSF vaccination significantly interfered with an induction of anti-CSFV immunity. Although the PRRSV infected group exhibited slight elevation of the mean anti-CSFV SN titer at the first week following vaccination, but the titers constantly declined afterward. By the time of CSFV challenge, the mean SN titer of the PRRSV-infected group (group D) was significantly lower than the non-infected, vaccinated group (group B). No priming effect on either cell-mediated or humoral immune responses was observed in the PRRSV infected, vaccinated group following the CSFV challenge. Furthermore, vaccination during an early stage of PRRSV infection did not protect nor reduce viremia or viral spreading in the challenged pigs. Moreover, remarkably high IFN-γ production by the PBMC, accompanied with secondary bacterial infection, suggesting that overwhelmed CSFV infection occurred in both PRRSV infected group regardless of CSF vaccination status. These findings corresponded with the previous finding that PRRSV infection affected the induction of humoral immune response to CSFV [25]. Furthermore, the effect extended to the cell-mediated immune response as well.

Interestingly, the PRRSV-infected pigs (group C and D) exhibited faster onset of clinical sign and were dead earlier than the non-infected group (group A). This result was in line with the previous report demonstrating that PRRSV infection could accelerate the progress of CSF [31]. There seemed to be prolonged PRRSV-viremia in the vaccinated pigs (group D) compared to the non-vaccinated group (table 2). PRRSV could be detected in the pigs from group D up to day 38 while the PRRSV titer in the group C was undetected by the time of challenge. At present, the reason for this is not clearly known. Further time-course study on the PRRSV titers in both serum samples and tissues will be needed to confirm this phenomenon.

PRRSV infection induces rapid onset of viremia within the first day of infection [32, 33], and viremia lasts up to 1 month [33, 34]. In general, pathogenesis of PRRSV can be defined into 3 phases. The early acute phase involves a rapid onset of viremia and systemic infection of the lymphatic organs. This process occurs during the first 2 weeks of infection, follows by the delayed acute phase that occurs primarily in the lungs during 2-3 weeks post infection. The persistent phase lasts several months after infection [35]. During the early acute phase, PRRSV

rapidly replicates in many cell types including monocytes/macrophages and dendritic cells of the lymphoid organs throughout the body [35, 36]. These cells have been known to involve in an induction of specific immune responses. Therefore, it is conceivable that PRRSV infection may post systemic effect on the immune system of the infected host during the early acute phase of infection. Supporting this notion, it has been previously shown that PRRSV infection can significantly affect the induction of immune responses by several vaccines including the CSF vaccine [23-25].

Previous reports demonstrated that PRRSV infection poorly induced innate immune responses followed by delayed viral-specific immunity, and impaired lung defense mechanism [15-17, 37, 38]. Suppression of T cell responses to PRRSV has been previously suggested [36, 39]. These findings lead to the concept that PRRSV may possess immunomodulatory properties. However, studies related to the immunomodulatory role of PRRSV often give contradicting results [14]. Several factors including stage of PRRSV infection at the time of study, immunological competency of the experimental pigs, and the interaction between PRRSV and the co-infecting pathogens or vaccines, could be responsible to the discrepancy among the observations from different studies.

Although PRRSV replication occurs in lymphoid tissues throughout the body during the acute phase of infection [35], significant cytopathic effect of the immune cells has not been reported in the lymphoid organs or the lungs. No significant changes in the numbers of macrophage, or alteration of the numbers of major T cell subpopulation were observed in blood and lymphoid tissues or lungs [36, 40]. These findings suggest that other mechanism of PRRSV-host interaction could be responsible to the unique immunological picture observed following PRRSV infection. Recently, several studies have shown that PRRSV infection, in particular during the active stage, resulted in systemic and local productions of a potent immunosuppressive cytokine, IL-10 [18-21]. In our experiences, not only the virulent PRRSV strain but the modified live PRRSV vaccine strain could also induce in vivo IL-10 production as well (S. Suradhat, unpublished observation). Interleukin 10 has been recognized as an important cytokine that downregulates functions of antigen presenting cells and several effector populations [41]. Moreover, it has been recently shown that IL-10 plays a crucial role in differentiation and function of regulatory dendritic cells (DCr) and facilitates differentiation of pathogen-specific regulatory T cells (Tr1) which downregulate the antigen-specific immune responses. Induction of IL-10 production during the active stage of infection has been known as one of the immune evasion strategies by several intracellular pathogens [42, 43].

We postulated that the early systemic induction of IL-10 could result in systemic inhibition of immune responses to other pathogens during the same period. Prior to the CSFV challenge, IL-10 production of the PRRSV infected groups, following in vitro culture with CSFV, fluctuated while no significant change was observed in the other groups (figure 3). The increased IL-10 production in the cultured cells peaked approximately 2 weeks following PRRSV infection. It is unlikely that the IL-10 production was CSFV-specific, since the non-vaccinated, PRRSV infected pigs (group C) also exhibited enhanced IL-10 production. Previously, it has been shown that the

presence of PRRSV in the culture resulted in enhanced IL-10 production by the CSF-primed PBMC [26]. The enhanced IL-10 production observed in the group C and D was likely due to the carried over PRRSV and/or infected PBMC into the culture system. In addition, increased IL-10 production in the unprotected groups following the CSFV challenge could relate to an overwhelmed CSFV infection. It has been previously shown that PBMC of the CSFV-infected unprotected pigs contained high numbers of IFN-γ and IL-10 producing cells, in particular in the CD8+ subpopulation, following the CSFV challenge [44]. However, it should be noted that the recovered pig (group D) still possessed high number of IL-10 producing cells by day 41, suggesting that systemic immuosuppression could still occurred in this pig. This information implied that the recovered animal might be vulnerable to the secondary infection in the field situation.

The result that PRRSV infection significantly inhibited the CSF vaccine efficacy confers several implications to the swine industry, in particular the CSFV-endemic countries with high prevalence of PRRSV. The findings from this study suggest that CSF vaccination during the active PRRSV infection should be avoided. It should be noted that the virulent strain CSFV (Bangkok 1950), used in this experiment, induced acute CSF that resulted in typical clinical signs of CSF with 100% mortality rate in a short period of time. However, with the increased prevalence of subacute and chronic CSF was recently observed in endemic countries particularly in Asia [6, 45], it is likely that CSFV-infected pigs will be able to survive for a longer period. Moreover, the clinical signs of CSF may be masked by other bacterial or viral complications in the field [46]. In this case, the infected pigs would serve as undetected source of CSFV infection in the farm for a long period of time and would certainly complicate the management and control strategies. Therefore, regular monitoring of the herd immunity against the CSFV and PRRSV infection status in the farm would be critical and strongly encouraged in the endemic areas.

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Legends

Figure 1 Total leukocyte count following the CSFV challenge. Experiment pigs were infected with PRRSV (PRRSV) on day 0 and vaccinated with CSF vaccine (Vac) a week later. Three weeks after CSF vaccination, the pigs were challenged with the virulent CSFV (Chall). Blood samples were collected and numbers of leukocyte count were determined at 0, 3, 7 and 14 post challenge. Naïve pigs were included in the study as a negative control. Data represents mean (±SD) of total leukocyte count. Group treatments were indicated in the legends. Leukocyte number of < 9,000 cell/mm^3 is considered leukopenic.

Figure 2 Levels of CSFV-specific serum neutralizing (SN) antibody titers. Pigs were infected with PRRSV (PRRSV) on day 0 (0) and vaccinated with CSF vaccine (Vac) a week later (7). Three weeks after vaccination (28), the pigs were challenged with CSFV (Chall). Group treatments were indicated in the legends. Data represent mean (±SD) of the log2 SN titers determined by NPLA.

Figure 3 Total IFN-γ (A) and IL-10 (B) production by porcine peripheral blood lymphocytes. Pigs were infected with PRRSV (PRRSV) on day 0 (0) and vaccinated with CSF vaccine (Vaccine) a week later. Three weeks after vaccination (28), the pigs were challenged with CSFV (CSFV). Porcine PBMCs were cultured in vitro with CSFV for 40 hr before harvesting for flow cytometric analyses. Group treatments were indicated in the legends. The data represent the mean (± SEM) of the % cytokine positive cells.

Figure 4 IL-10 productions by porcine peripheral blood lymphocyte subpopulations. IL-10 productions by lymphocyte subpopulations from the pigs that were vaccinated with CSF vaccine (A), and the pigs that were infected with PRRSV (day 0), prior to the CSF vaccination (B). Both groups were challenge with CSFV on day 28. The studied lymphocyte subsets included $CD4^+CD8^-$ ($CD4^+$), $CD4^-CD8^+$ ($CD8^+$) and $CD4^+CD8^+$ populations.

Table 1 Experimental protocol and number of survived pigs

Gr.	Treatments			(PRRSV)	(Vaccine)	(CSFV)			Survival
	PRRSV	Vaccine	CSFV	D0	D7	D28	D35	D41	rate[c] (%)
A	**-**	**-**	**+**	5	5	5	5	0	0
B	**-**	**+**	**+**	5	5	5	5	5	**100**
C	**+**	**-**	**+**	7	7	4[a]	1	0	0
D	**+**	**+**	**+**	6	6	5[b]	2	1	**20**
E	**-**	**-**	**-**	5	5	5	5	5	100

[a] Three pigs died from *Haemophilus spp.* infection on D 10, 11 and 18.

[b] One pig died from *Haemophilus spp.* infection on D12.

[c] Survival rates were calculated from (the number of pigs on D41 / the number of pig on D28) x100.

Table 2 PRRSV and CSFV titers from pooled serum samples collected during the experiment

	D0 (P)	D7 (V)	D14	D28 (C)	D31	D33	D35	D38	D41
PRRSV titer[a]									
A (- / - /C)[d]	-[b]	-	-	-	-	-	-	-	na[c]
B (- / Vac /C)	-	-	-	-	-	-	-	-	-
C (P/ - /C)	-	3.5	<2	-	-	-	-	na	na
D (P/ Vac /C)	-	3.25	3	2.5	2.5	2.25	2.5	-	-
E (- / - / -)	-	-	-	-	-	-	-	-	-
CSFV titer									
A (- / - /C)	-	-	-	-	3.63	6.97	7.97	-	na
B (- / Vac /C)	-	-	-	-	-	-	-	-	-
C (P/ - /C)	-	-	-	-	4.80	6.30	7.80	na	na
D (P/ Vac /C)	-	-	-	-	4.63	6.97	6.97	2.97	-
E (- / - / -)	-	-	-	-	-	-	-	-	-

[a] viral titers from pooled serum sample (log$TCID_{50}$/ml)
[b] Not detected
[c] Not applicable due to animal death
[d] Treatments: P; PRRSV, C; CSFV challenge, V; CSFV Vaccine

Table 3 Classical swine fever virus titers from internal organs of the experimental pigs at the end of the experiment (or at the time of death)

Gr. (treatment)	Viral titers (log $TCID_{50}$/gram)				
	A (-/-/C)	**B** (-/V/C)	**C** (P/-/C)	**D** (P/V/C)	**E** (-/-/-)
Brain	4.13 ± 0.60*[a]	0[b]	4.80 ± 0.71[a]	2.80 ± 0.17[c]	0[b]
Tonsil	4.08 ± 0.19[a,b]	0[c]	4.55 ± 1.77[a]	3.25 ± 0.58[b]	0[c]
Lung	4.32 ± 0.62[a]	0[b]	3.72 ± 1.47[a,c]	2.66 ± 1.05[c]	0[b]
Spleen	3.84 ± 0.63[a]	0[b]	4.11 ± 2.26[a]	4.06 ± 1.06[a]	0[b]
Kidney	3.81 ± 0.83[a]	0[b]	5.14 ± 0.23[c]	3.45 ± 0.89[a]	0[b]
Lymph nodes	3.89 ± 0.63[a]	0[b]	5.22 ± 0.83[c]	3.36 ± 0.42[a]	0[b]

* Data represents mean ± SD virus titers (log $TCID_{50}$/gr)
[a-c] indicates statistical differences among the experimental groups (ANOVA, $p<0.05$)
Treatments: P; PRRSV, C; CSFV challenge, V; CSFV Vaccine

Table 4 Percentages of experimental pigs exhibiting pathological changes that related to CSF

	Experimental group (treatments)				
	A (-/-/C)[a]	**B** (-/V/C)	**C** (P/-/C)	**D** (P/V/C)	**E** (-/-/-)
Pneumonia	100[b]	0	100	100	0
Lymphadenopathy					
- inguinal ln.	75	0	75	75	0
- tracheobroncheal ln.	75	0	100	75	0
- mandibular ln.	100	0	100	100	0
- mesenteric ln.	40	0	40	40	0
Thymic atrophy	100	0	100	100	0
Haemorrhage					
- skin	60	0	40	20	0
- lymph node	60	0	40	40	0
- kidney	60	0	20	60	0
- tonsil	0	0	0	20	0
- gastric mucosa	0	0	20	0	0
Splenic infarction	0	0	20	75	0
Brain congestion	100	0	100	100	0
Gastric infarction	20	0	20	0	0
Fibrinous pleuritis	0	0	20	0	0
Fibrinous peritonitis	0	0	20	0	0

[a] Treatments: P; PRRSV, C; CSFV challenge, V; CSFV Vaccine
[b] Percentages of animals with CSF lesion

Table 5 Anti-PRRSV antibody responses and percent PRRSV-seropositive pig during the experiment

GROUPS (Treatment)	Mean S/P ratio[a] (% pig positive)						
	D0 (P)	**D7 (Vac)**	**D14**	**D21**	**D28 (C)**	**D35**	**D38**
A (- / - /C)[c]	0	0.020	0.016	0.010	0.018	0.017	0.050
	(0)	(0)	(0)	(0)	(0)	(0)	(0)
B (- / V/C)	0	0.035	0.012	0.092	0.011	0.041	0.009
	(0)	(0)	(0)	(0)	(0)	(0)	(0)
C (P/ - /C)	0.003	0.157	**1.095**	**1.838**	**1.440**	**1.306**	na[b]
	(0)	(0)	(80)	(100)	(66.67)	(66.67)	
D (P/ V /C)	0.002	0.273	**0.959**	**1.016**	**0.745**	**1.061**	**2.379**
	(0)	(16.67)	(60)	(60)	(75)	(100)	(100)
E (- / - / -)	0.050	0.016	0.032	0.108	0.010	0.009	0.008
	(0)	(0)	(0)	(0)	(0)	(0)	(0)

[a] Cut-off value for PRRSV-seropositive when S/P ratio $\geq$ 0.4 (IDEXX® ELISA test kit)
[b] not applicable due to animal death
[c] Treatments: P; PRRSV, C; CSFV challenge, Vac; CSFV Vaccine

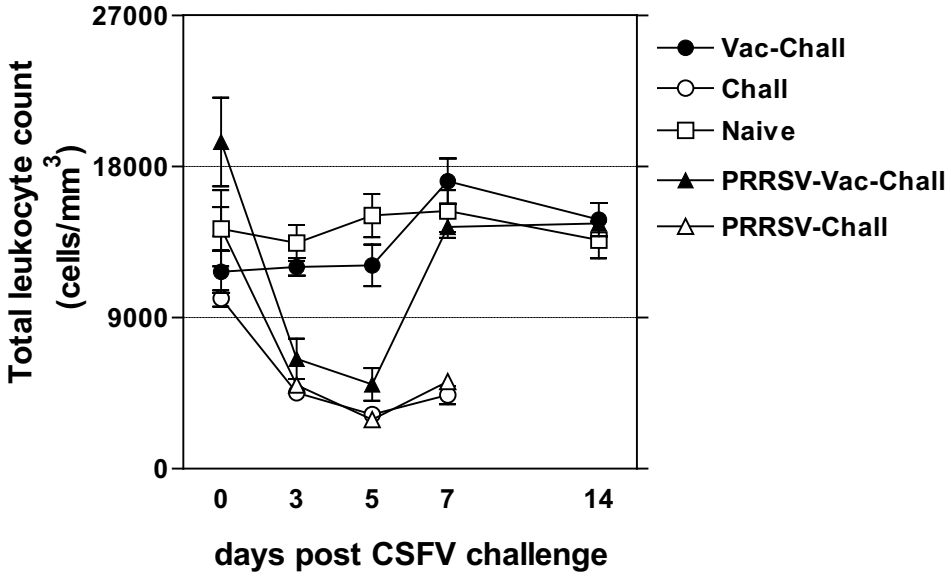

Figure 1 (Suradhat et al.)

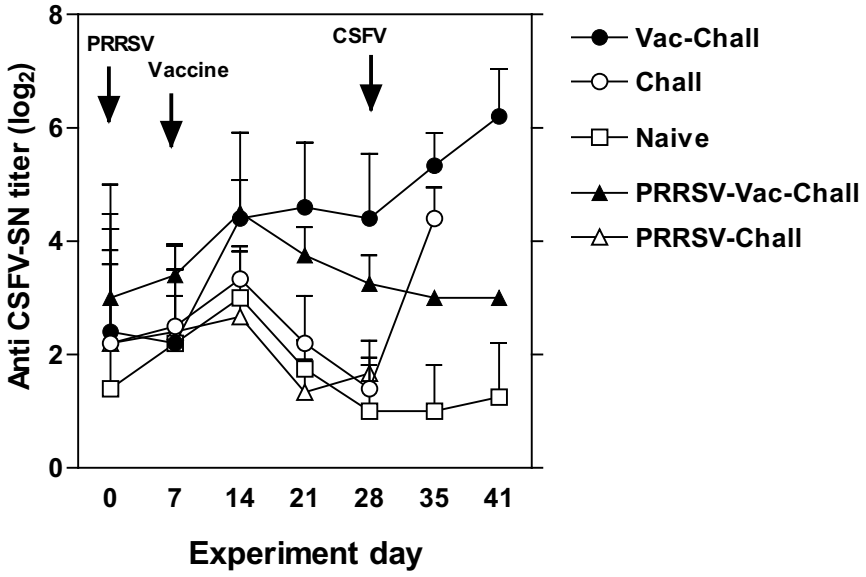

Figure 2 (Suradhat et al.)

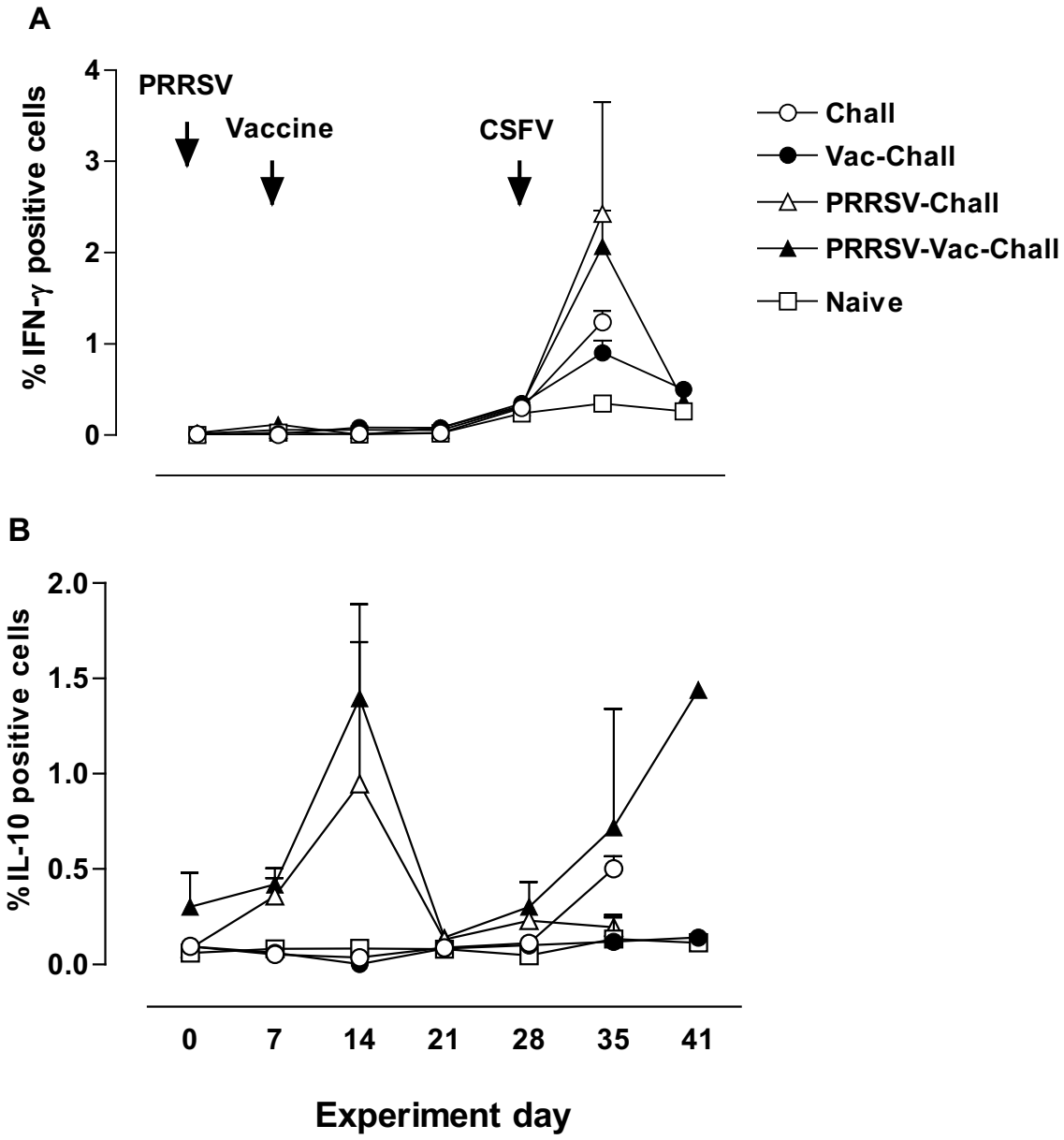

Figure 3 (Suradhat et al.)

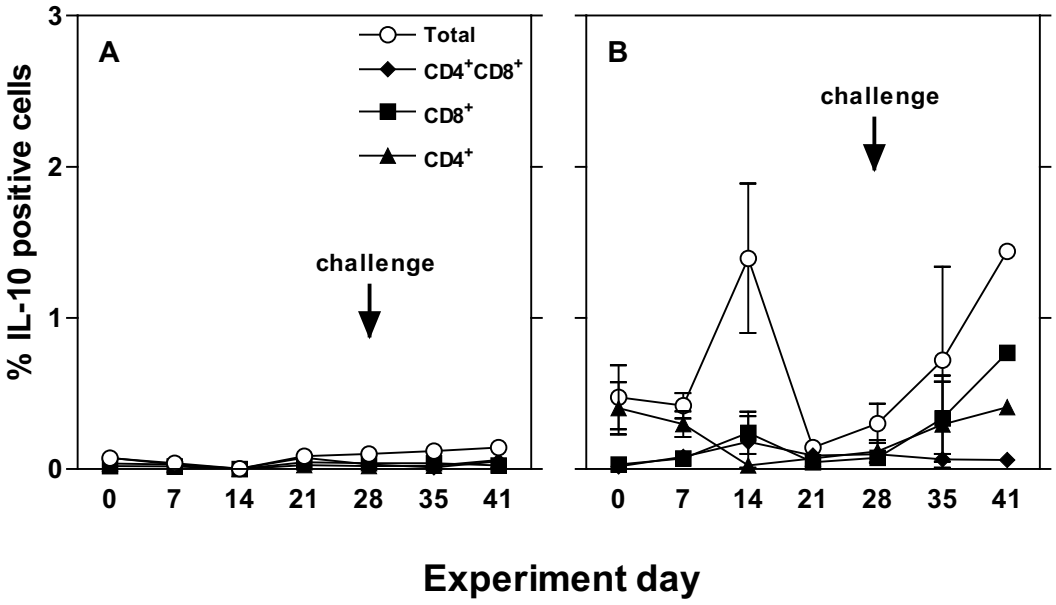

Figure 4 (Suradhat et al.)

Classical swine fever handbook

Classical swine fever vaccine: the role of cell mediated immunity and other factors on induction of protective immunity

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Summary

Classical swine fever (CSF), caused by classical swine fever virus (CSFV) is one of the most important viral diseases in the swine industry worldwide. During the past 5 years, several techniques for measuring of porcine cell-mediated immunity (CMI) were established and applied in conjunction with other conventional assays, to study factors that influence the induction of anti-CSFV immunity. The studies emphasized the role of cell-mediated immunity in protection against CSFV infection. Although our results showed that the available modified live CSF vaccines in Thailand could induce complete heterotypic protection, several factors including maternal immunity, age at primary vaccination, vaccination protocol and complication by other pathogens, could greatly affect the effectiveness of CSF vaccines in the field.

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Classical swine fever (CSF) is one of the most important diseases in pigs, causing serious economic losses to the swine industry worldwide [1]. Despite tremendous efforts to control the disease during the last decade, CSF continues to cause problems in Thailand and Southeast Asian countries. Classical swine fever virus (CSFV) is an enveloped, single-stranded RNA virus in the genus Pestivirus of the *Flaviviridae* family [2]. Recently, several new genogroups of CSFV have been identified; some are unique to the Asia region [3, 4]. The newly emerged genogroups have become the major strains circulated in the area, causing a milder clinical symptoms ranged from subacute to chronic forms of CSF outbreaks [5, 6].

In the endemic area, routine vaccination is one of the most effective strategies for prevention and control of CSF. However, the increased incidence of subacute CSF during the 90's in Thailand raised several concerns whether the vaccines and vaccination programs were still effective in preventing of CSF in the country. Since the year 2000, we have carried out a research program conducting several studies to assess the efficacy of the CSF vaccines and evaluate factors affecting them. The information from the research program will be further discussed in this article.

Detection of CSFV-specific cell-mediated immune response

Previously, the assessment of anti-CSFV immunity primarily relied on detection of serum neutralizing (SN) antibody of the pigs. However, measuring of SN titers does not allow differentiation between active and passive antibodies, in particular when conducting a vaccine efficacy testing in young pigs with certain level of maternal derived antibody (MDA). In addition, CSFV-specific SN antibodies are usually not detected prior to 2-3 weeks post vaccination. Therefore, in the early challenge study, the evaluation of active humoral immune responses in association with disease protection was not possible, as the challenged pigs are usually died within the first 2 weeks.

Cell-mediated immunity (CMI) is known to have a direct regulatory role on immune responses and is believed to be essential for immunity against intracellular pathogens, including viruses [7]. Previous evidence regarding the protective role of CMI in CSFV-infected pigs is very limited. Nevertheless, antigen-specific lymphoproliferative activity was demonstrated in peripheral blood lymphocytes from vaccinated pigs that were protected against CSFV challenge [8]. In addition, the protective role of cytotoxic T lymphocytes (CTL) has been confirmed in several studies [9-11]. However, the assays used for studies of porcine cellular effector functions are rather laborious, expensive, and not practical for the field trial.

An alternative technique for assessing CMI is to measure the production of cytokines that are known to influence or directly relate to cellular immune responses. Among the cytokines, the role of IFN-γ for induction of CMI has been well characterized. IFN-γ has several effector functions and immunoregulatory roles that involve in the induction of anti-viral immunity, including the activation of CTL, natural killer (NK) cells and phagocytes [7]. Recently, an ELISPOT assay for measuring porcine IFN-γ was established, and IFN-γ was demonstrated as a good indicator of anti-viral immunity in pigs [12]. In addition, the measurement of IFN-γ was

found useful for detecting the presence of antigen-specific, immunological memory over lymphoproliferative assay in pigs. Detection of antigen-specific IFN-γ has been used for assessing the CMI of pigs in a pseudorabies virus (PRV) model [13, 14]. From these evidences, we set the first objective to establish and validate an ELISPOT assay for detection of CSFV-specific IFN-γ producing cells from porcine peripheral blood mononuclear cells (PBMC). The basic principle of the assay, is to detect and enumerate the number of IFN-γ producing cells from the CSFV-stimulated PBMC, i.e. measuring of the CSFV-specific memory T cell [15]. The ELISPOT assay has been successfully established and applied, in conjunction with the viral neutralization assay, to evaluate the efficacy of CSF vaccine and vaccination protocols [15-17]. In addition, we recently established the intracellular cytokine staining technique in which the cytokine producing lymphocyte subpopulation could be further characterized by flow cytometry [18]. The information obtained from these assays will be helpful for a better understanding of the mechanisms required for induction of protective immunity against CSFV and in evaluating the new generation of CSF vaccine in the future.

CSFV-specific cell-mediated immunity and its role in disease protection

CSFV is a cell-associated, and non-cytopathic virus. The replication is restricted in the cytoplasm of the cell and the virus can spread directly from the infected cell to the adjacent cells [19]. This information suggested that both arms of the immune response will be required to achieve complete protection of the infected pigs. Cell-mediated immunity is critical for eliminating viral infected cells and limiting viral spreading in between the cells, while the humoral immunity helps reduce spreading/shedding of the infectious virions.

In recent years, a number of studies have focused on the mechanisms of protective immunity to CSFV. In some cases CMI was implicated in protection, in the absence of neutralizing antibodies [20, 21]. Our finding is also consistent with the previous reports. Following a single vaccination with a modified live CSF vaccine, cell-mediated immune response measuring by the ELISPOT assay was detected as early as 6 days and lasted up to 140 days following vaccination [16]. At day 6 post vaccination, the vaccinated pigs contained significantly higher numbers of the CSFV-specific IFN-γ secreting cells, than the unvaccinated pigs. When challenged with the virulent CSFV on the same day, these pigs were completely protected while all of the unvaccinated pigs died within 2 weeks post infection. It should be noted that the complete protection was achieved in the absence of detectable CSFV-specific neutralizing antibodies. Furthermore, the role of CMI in protection against CSFV infection was also indicated even in the presence of CSFV-specific neutralizing antibodies. In one of our challenge experiments, when the pigs were challenged at 140 days after a single vaccination, good correlation between the level of protection and the level of IFN-γ production, rather than the antibody titer, could still be demonstrated.

Our findings emphasize the significant role of CMI in protection against CSFV. The evidence that the level of viral-specific IFN-γ production, but not antibody response, conferred resistance against PRV challenge was also demonstrated [13]. These results also explain why

the previously available inactivated vaccine, which does not efficiently induce CMI, was not effective in controlling of CSF and PRV infections. It should be also mentioned that, in general, the CSFV-specific SN titer correlates well with the levels of IFN-γ production following vaccination with the modified live vaccine (MLV) [16]. Therefore, routine monitoring of SN titers, i.e. seroprofile, can still be used as an indicator for assessing the effectiveness of CSF vaccine program in the field. Indeed, we strongly recommend evaluation of the status of herd immunity to CSFV as a routine practice for the farms in highly endemic zone.

Factors affecting the effectiveness of CSF vaccines

The vaccines

Recently, several novel CSF vaccines accompanied with the diagnostic tests, allowing differentiation of infected from vaccinated animals (DIVA) vaccine strategy, have been developed. The detail information with regard to efficacy and safety of these vaccines are extensively reviewed elsewhere [22]. At present, the modified live CSF vaccines have been proven to efficiently induce both CMI and humoral immunity while the subunit vaccines primarily induce humoral immunity in vaccinated animals [22]. Our research work during the past years has focused primarily on the evaluation of factors affecting the effectiveness of the CSF-MLV.

The modified live, C-strain, CSF vaccine is regarded as one of the most effective CSF vaccines that provide not only clinical but also complete virological protection, i.e. sterile immunity, within a week following vaccination [16, 22]. In fact, it has been suggested that the modified live C-strain may be the vaccine of choice in an emergency vaccination protocol [22]. Nevertheless, several strains of modified live vaccines are currently available on the Thai market. In our experiences, all of the tested vaccines including Chinese (both lapinized and tissue culture derived), and GPE^- strains induced complete protection against CSFV infection in vaccinated pigs as early as 6 days following a single vaccination. It should be emphasized that the result was obtained under the condition that the pigs had no interfering effect form MDA at the time of vaccination.

Several genogroups of CSFV have been recently isolated in Thailand [4]. Although it is well accepted that the CSF vaccine effectively induces protection against homotypic and heterotypic CSFV strains, an increased prevalence of the newly emerged genogroups during the last decade has raised the concern whether the available vaccines can induce complete protection to all genogroups. Our challenge studies, together with the other previous reports in Thailand, confirmed that the CSF vaccine induced complete protection against the CSFV, including the newly emerged genogroup 2 [4, 23]. Therefore, the increased prevalence of the new genogroups is unlikely due to the inability to induce heterotypic protection by the CSF-MLV.

Although, our group and others have shown that the C-strain MLV effectively induce CSF protective immunity in immunized pigs. It should be noted that certain conditions are required to achieve optimal protection by CSF vaccination (see below). The efficacy of extra-label vaccine usage, for example vaccination in combination with other vaccines or oral vaccination with the vaccine registered for systemic administration, should be verified only by the challenge study. In

one of our studies, while no difference was observed in the levels of serum neutralizing antibody, a combination of C-strain MLV with a live gE-deleted PrV vaccine, resulted in significant reduction of cell-mediated immune response against CSFV, and subsequently induced less protection [16]. Although oral vaccination has been discussed in the European countries for the purpose of controlling CSF in wild boars [22], it should be noted that vaccine stabilizer and a higher dose of the vaccine virus are required to obtain such protection in the vaccinated boars [24]. More information on protection study will be needed to evaluate the effectiveness of this vaccination regimen in domestic pigs. Thus, oral vaccination using commercially available CSF-MLV in domestic pigs is not recommended at the moment.

The pigs

From our experiences during the CSF research program, MDA is regards as the most common factor affecting an induction of protective immunity in vaccinated piglets in the field. Piglets are born agammaglobulinaemia and acquire early passive immunity via colostrum. Thus, the levels of MDA in piglets correlate well with the levels of SN titer in sows and, in case of CSFV, gradually decline with a half-life of approximately 2 weeks [25]. Generally, it is well accepted that optimal protection can be achieved by vaccination of the pigs with MDA titers of $\leq$ 1:32 [26, 27]. This can be problematic in the highly endemic area where natural exposure in the sows, resulting high level of anti-CSFV SN titers, can occur unnoticeably. Although high levels of MDA may confer some degrees of protection in the piglets, in our experience, even the MDA titer of 1:128 could not protected the pigs from CSFV challenge in our model. One previous report demonstrates that the MDA titers of more than 1:256 is required to obtain complete protection in piglets [27]. Furthermore, it takes longer time for the MDA level to decline to the level that does not interfere the effectiveness of CSF vaccination, i.e. longer window of susceptibility. Our recent result also supports the previous work, in which MDA of more than 1:32 significantly interfered the induction of protective immunity by CSF-MLV [23]. It should be noted that in some experiments, piglets that were vaccinated in the presence of high MDA titers never showed seroconversion [27], or anamnestic response to the CSFV challenge [23]. Although, these pigs survived the challenge and the protective value of CMI was implicated, it was unlikely that complete virological protection, i.e. protection of viremia and viral shedding, would be achieved in the absence of humoral immunity. Moreover, with the increased prevalence of subacute and chronic CFV cases during the past years, infected pigs may survive for a longer period of time without obvious clinical signs of CSF infection. One of our challenge studies, using the moderate virulent CSFV, for the challenge virus, clearly showed that primary immunization of the pigs with high MDA titers resulted in vaccine failure. However, all the challenged pigs survived the CSFV infection and viremia was observed in both control and vaccinated, with high MDA titer, groups. This picture will certainly complicate the disease control program as the infected pigs would become the undetected source of infection in the farm. Together, these findings highlight the significance of routine serosurveillance and the use of other laboratory tools for monitoring of the herd status.

Apart from MDA, the influence of age of the piglets at the time of primary immunization was also investigated. We conducted the experiment exploring the influence of the pig age at primary immunization on the effectiveness of a CSF vaccine. Piglets at the age of 3-5 weeks, with comparable levels of MDA titers, were immunized twice at 2 weeks interval with the lapinized C-strain MLV. Both cell-mediated and antibody immune responses were followed up to 10 weeks post vaccination. The result clearly showed that pigs immunized at the older age had more numbers of CSFV-specific IFN-γ producing cells in the PBMC and higher CSFV-specific SN titers at the end of the experiment [17]. Although CSFV challenge was not included in this study, the results clearly demonstrated that at the age of 3-5 weeks, younger pigs are less immunocompetent than the older ones. The finding is also consistent with the previous knowledge that porcine immune system is fully matured at the age of 4 weeks [28]. This information should be kept in mind when emergency vaccination is implemented, under professional discretion, to the very young, i.e. suckling, pigs. It is highly recommended that the emergency vaccination protocol should be adjusted back to the normal vaccine protocol once the crisis is over.

Complication by other pathogens

As shown through our series of experiment, induction of protective immunity against CSFV infection depends on several factors. It should be pointed out that most of the challenge studies were conducted in isolation units where disease complications by other pathogens were minimized. The results and interpretations obtained from these experiments could underestimate the clinical outcomes that actually occurred in the field, where pigs can be exposed to several pathogens at the same time. Several pathogens, mycotoxins and chemicals were known to negatively modulate the immune system, and, therefore can significantly interfere the effectiveness of CSF vaccination protocol. In our experience, coinfection of PRV at the time of CSFV challenge resulted in fatal infection in vaccinated pigs, despite the successful immunization against CSFV prior to CSFV challenge (S. Suradhat, unpublished observation). Therefore, the influence of other pathogens on the immune system and/or interaction with CSFV also needed be taken into consideration in the field practice. Improvement of biosecurity and routine monitoring of the herd status will be crucial for preventing of such complications.

Since its emergence in the late 1980's, porcine reproductive and respiratory syndrome virus (PRRSV) has become one of the most economically important pathogens of the swine industry [29]. In Thailand, the prevalence of PRRSV infection is believed to be more than 80% [30]. Several lines of evidence suggested that PRRSV could negatively modulate the immune system. Our recent findings demonstrated that PRRSV induced a very potent immunosuppressive cytokine, interleukin-10 (IL-10), during an early stage of infection [31]. Furthermore, the presence of PRRSV in the culture suppressed IFN- production by the CSFV-primed PBMC when stimulated with the recall antigen [32]. Recently, we conducted the CSFV challenge experiment in order to explore the effect of PRRSV infection on the efficacy of CSF vaccine. Seventeen days old pigs were infected with the Thai PRRSV isolate a week prior to vaccination with the CSF-MLV. Three weeks after vaccination, the pigs were challenged with a

virulent CSFV. The results demonstrated that PRRSV infection significantly interfered the induction of CSFV-specific immune responses, and resulted in vaccine failure (Suradhat et al., manuscript in preparation). This finding implied that CSF vaccination during the active stage of PRRSV infection should be avoided.

Final remarks

During the past few years, we have explored several factors that could influence the effectiveness of CSF vaccines in the field. Our results, together with the previous reports, affirm that the available CSF vaccines, when used properly, can effectively induce protective immunity against CSFV infection. In our opinion, the CSF vaccine failure observed in the field is primarily due to the lack of understanding in epidemiology, mechanisms of immunological protection, pathogenesis, and the importance of biosecurity. Sharing of the information among the veterinarians and farmers, and strengthen of the disease surveillance program will be required for a successful CSF preventive program which, in our hope, will eventually lead to eradication of CSF.

Acknowledgement

The authors wish to thank Dr. Ratree Platt for helpful comments during the manuscript preparation.

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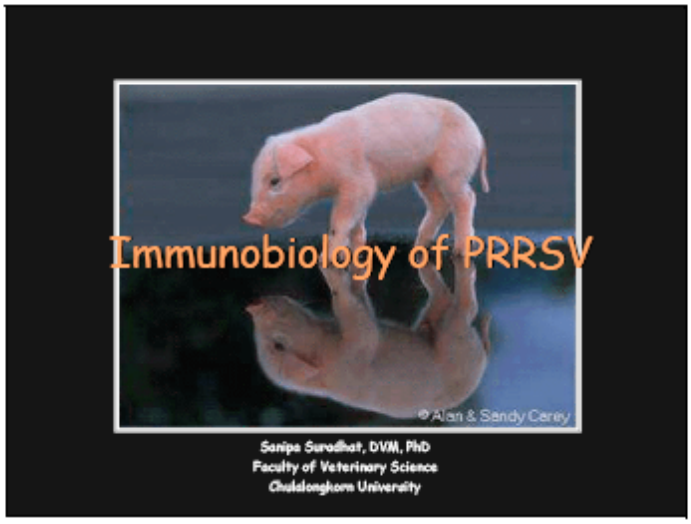

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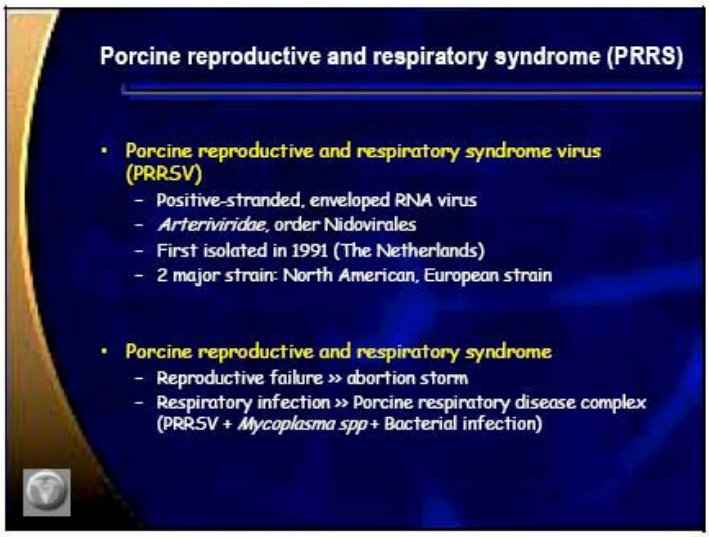

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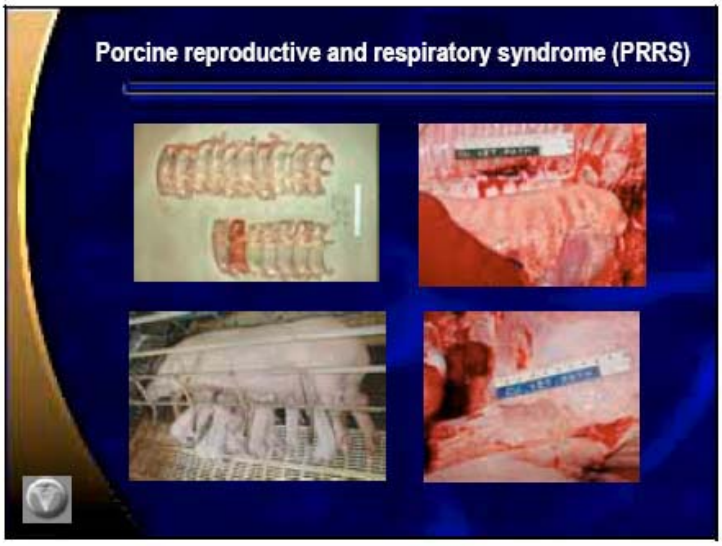

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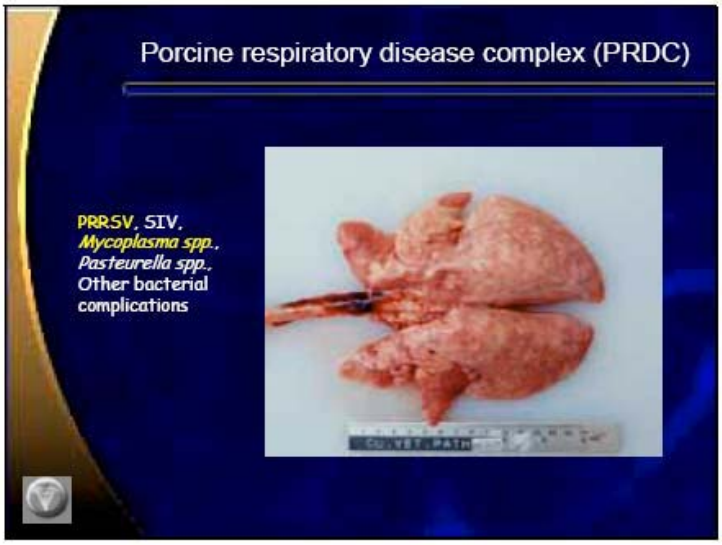
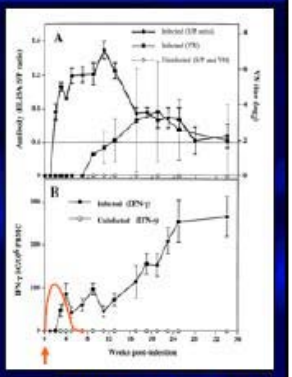


Slide 7

Immunobiology of PRRSV

- Primary target cells: alveolar macrophages and cells of the monocyte lineage
- Persistent infection (up to 150 days)
- Poor proinflammatory cytokine (IFN-α, TNF) production following infection
- Slow and weak CMI and Neutralizing Ab production
  - Early non-neutralizing antibody production results in antibody-dependent enhancement (ADE)
- Immunosuppression in the lungs following viral infection >> PRDC.



Meier et al., 2003. Virology. 309:18-31.

Slide 8

Immunobiology of PRRSV

- Both virulent PRRSV and MLV exhibit the same characteristics.
- Addition of adjuvant did not help enhancing the anti-PRRSV immunity.
- Intrinsic properties of the virus delays the development of IFN-γ response and preferentially stimulates the synthesis of non-neutralizing antibodies.

Meier et al., 2003. Virology. 309:18-31.

Slide 9

Techniques for studying of CMI

Activated Ag-specific cells

- Upregulation of surface molecule (cytokine receptor/MHC molecules etc)
- Cytokine production

RT-PCR
QPCR
Microarrays

>> detection of mRNA
>> detection of protein

ELSPOT
ELISA
IFA
Flow cytometry
(surface staining/ICCS)

Slide 10

Objectives

To study an *in vitro* effect of porcine reproductive and respiratory syndrome virus (PRRSV) on the level of cytokine production by porcine peripheral blood mononuclear cells (PBMC), using the multiplex-PCR assay

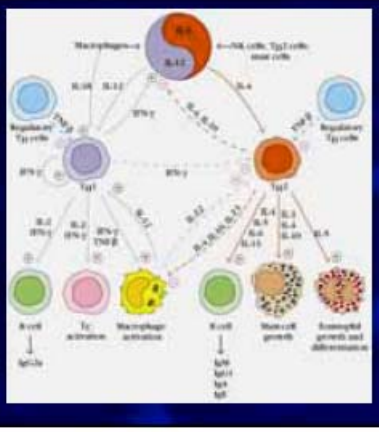
Duration: 1 yr (August 2001- August 2002)

Slide 11

Th dichotomy

Selected gene for mRNA studies (MPCR)

Th1: IFN-γ, IL2
Th2: IL4, IL10
Control gene: GAPDH



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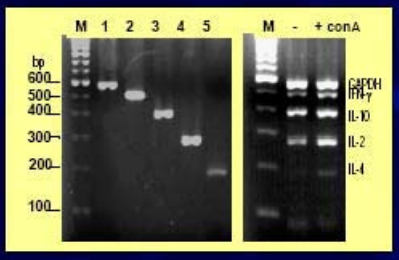
Specific aim 1: Establishment of the multiplex PCR assay

- Multiplex PCR that simultaneously analyses IFN-γ, IL-2, IL-4, IL-10 and GAPDH gene expression was established.
- Products were confirmed by sequence analysis.



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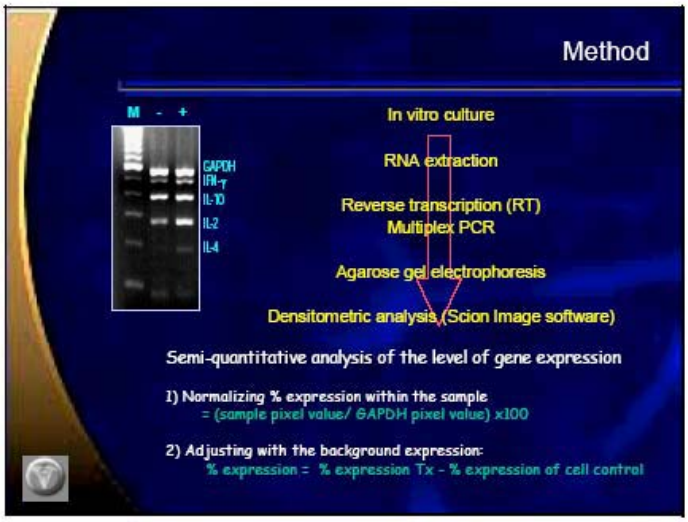

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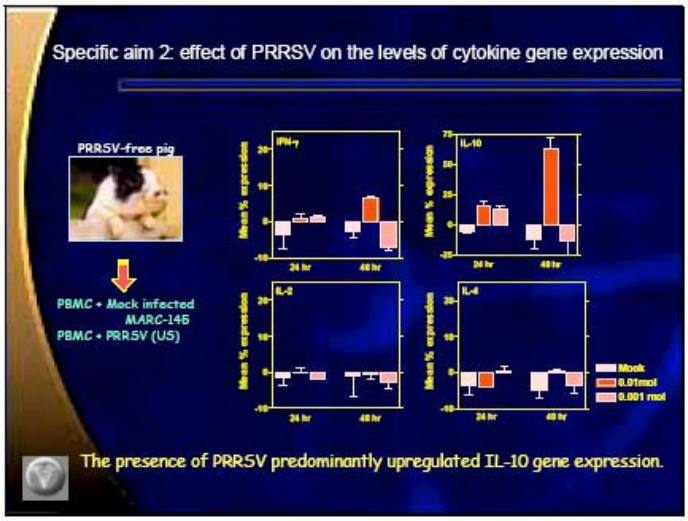

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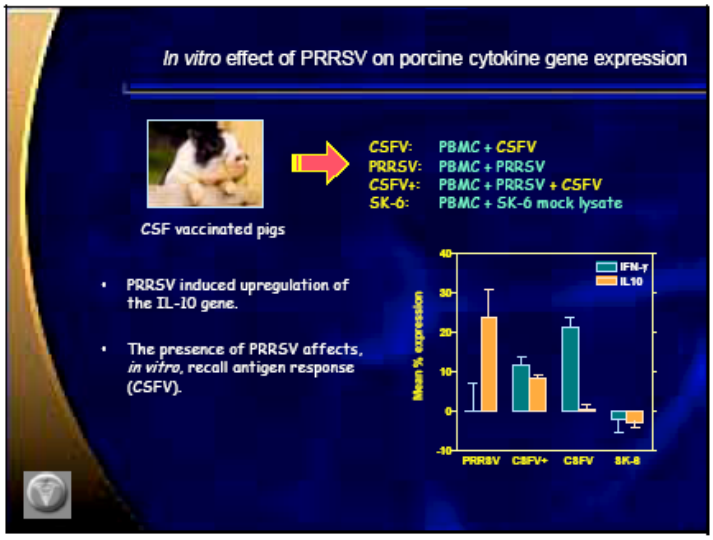

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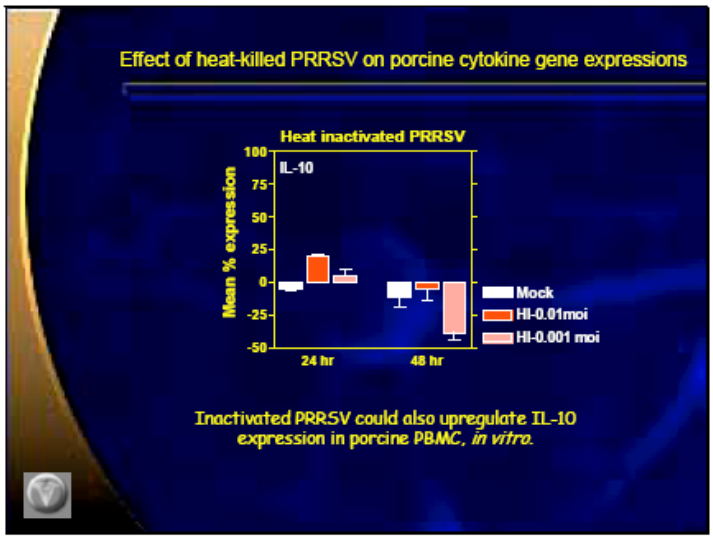

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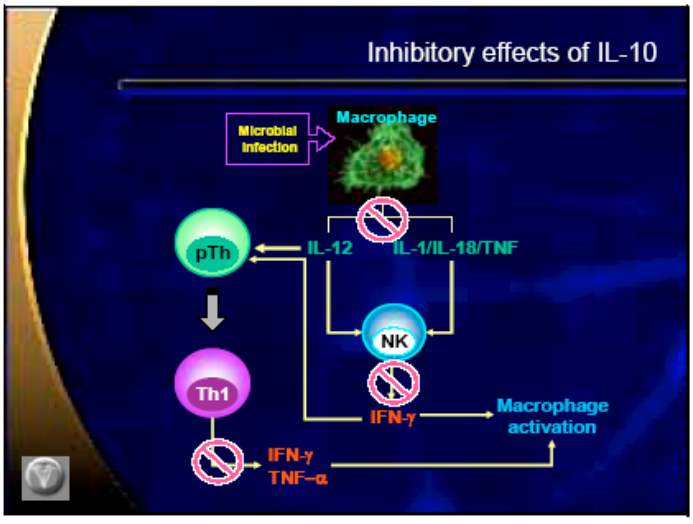

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Conclusion & discussion

- We established a multiplex PCR that allowed a semi-quantitative analysis of the IFN-γ, IL-2, IL-4, IL-10 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene expressions from porcine peripheral blood mononuclear cells (PBMC).
- Both live and inactivated PRRSV predominantly upregulated IL-10 gene expression in porcine PBMC.
- The presence of PRRSV in the culture could affect the recall antigen response.

Our study implies that the induction of IL-10 production may be one of the strategies used by PRRSV to modulate the host immune responses.

(Suradhat et al. J.Gen.Virol. 2003. 84:453-459.)

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In vivo effect of PRRSV on IL-10 gene expression

Thai isolate PRRSV
1) US strain (01NP1)
2) EU strain (02SB3)
3) control (uninfected pigs)

Day 0 5 9 12 15

Sample collection

- PBMC (5, 12 dpi) >> MPCR
- BAL (5, 9, 15 dpi) >> MPCR, IFA, differential cell count

- serum
- viral isolation
- lung score/clinical signs
- etc.

Rachadapisakesompoch Endowment fund 2002-3

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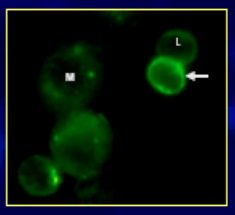
In vivo effect of PRRSV on IL-10 gene expression

- Upregulation of IL-10 gene expression was observed, *in vivo*, earlier in the PBMC (5 dpi), followed by in the BAL (9 dpi).
- Despite the differences in disease outcomes, both N. American and EU PRRSV comparably upregulate IL-10 gene expression in the leukocytes of infected pigs.
- Peripheral leukocytes may influence the level of cytokine production in the lungs of infected pigs.



IL-10 producing cells in BAL (IFA)

Suradhat & Thanawongnuwech. J. Gen. Virol. 2003. 84:2755-2760.
Thanawang et al. Thai J.Vet. Med. 2004. 34:29-38.

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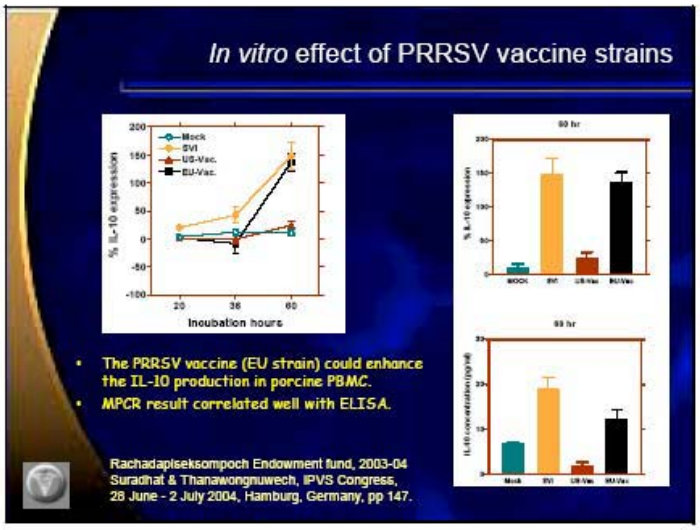

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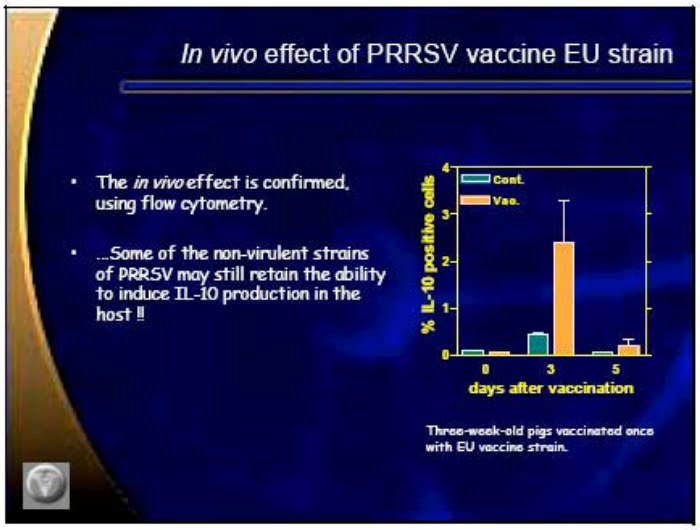

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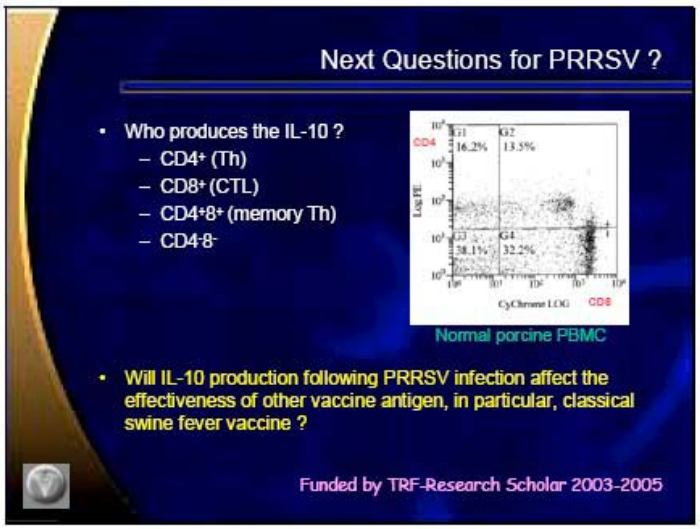

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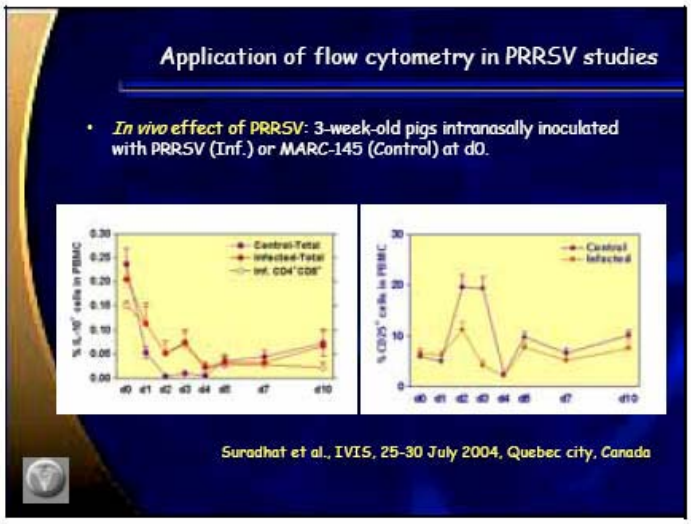

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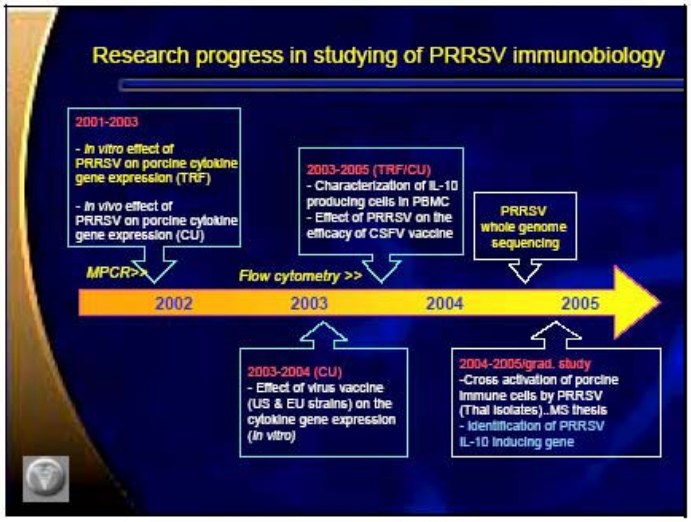

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The kinetics of cytokine production and CD25 expression by porcine lymphocyte subpopulations following exposure to classical swine fever virus (CSFV)

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Abstract

Surface expression of IL-2R-alpha (CD25) is widely used to identify activated lymphocyte populations, while interferon-gamma (IFN-γ) levels have been shown to be a good indicator of cell-mediated immunity (CMI) in pigs. To investigate the relationship between these two parameters, we developed an intracellular cytokine-staining assay and studied the kinetics of cytokine (IFN-γ and interleukin-10, IL-10) production relative to CD25 expression in porcine lymphocyte subpopulations, following immunization with a classical swine fever (CSF) vaccine. The number of activated memory T cells ($CD4^+CD8^+CD25^+$ cells) increased slightly in the peripheral blood mononuclear cell (PBMC) population soon after vaccination, then diminished within a few weeks. The number of activated cytotoxic T cells ($CD4^-CD8^+CD25^+$ cells) peaked approximately 2 weeks after the memory population. Although the number of IFN-γ producing cells detected in this experiment was relatively low, the $CD4^+CD8^+$ T cells were major IFN-γ producers in the PBMCs throughout the experiment. In another experiment, CSF-vaccinated pigs were challenged with a virulent classical swine fever virus (CSFV), and the kinetics of CD25 expression and cytokine productions were monitored. Following exposure to the virus, the number of IFN-γ producing cells in the PBMCs increased markedly in both the vaccinated and unvaccinated groups. The $CD4^-CD8^+$ cells were major IFN-γ producing cells in vaccinated pigs, while both $CD4^+CD8^+$ and $CD4^-CD8^+$ populations contributed to the IFN-γ production in the control group. Interestingly, the enhanced IFN-γ production was not associated with the upregulation of CD25 expression following the CSFV challenge. In addition, exposure to the virulent CSFV significantly increased interleukin-10 production by the $CD4^-CD8^+$ populations in PBMCs of the unvaccinated pigs. Taken together, our results indicated that CD25 expression and IFN-γ production were not tightly associated in porcine lymphocytes.

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In addition, the $CD4^{-}CD8^{+}$ lymphocytes of the PBMCs played a major role in cytokine productions following the CSFV challenge.

Keywords: Classical swine fever virus; Interferon-gamma; Interleukin-10; CD25; PBMC

1. Introduction

Classical swine fever (CSF) is one of the most important diseases in pigs, causing serious economic losses to the swine industry worldwide. The classical swine fever virus (CSFV) is an enveloped, single-stranded RNA virus in the genus *Pestivirus* (Moennig, 2000). In recent years, there have been a number of studies on the mechanisms of protective immunity to CSFV (reviewed in Van Oirschot, 2003). There seems to be a good correlation between the production of serum neutralizing antibodies and protection from disease. However, in some cases cell-mediated immunity (CMI) was implicated in protection, as neutralizing antibodies were absent (Launais et al., 1978; Rumenapf et al., 1991; Suradhat et al., 2001). T cell responses to CSFV in pigs have reportedly been absent or difficult to detect, and direct evidence regarding the protective role of CMI in CSFV-infected pigs is very limited. Nevertheless, antigen-specific lymphoproliferative activity has been demonstrated in peripheral blood lymphocytes from vaccinated pigs that were protected against CSFV challenge (Remond et al., 1981). In addition, the role of cytotoxic T lymphocytes (CTL) has been confirmed in several studies (Pauly et al., 1998; Armengol et al., 2002; Piriou et al., 2003). However, studies of porcine cellular effector functions are laborious and mostly limited to the inbred minipig model.

The cytokines and other factors associated with CMI have been identified in most species, including pigs (Wood and Seow, 1996; Pescovitz, 1998). Among these, the role of interferon-gamma (IFN-γ) in the induction of CMI responses is well characterized. IFN-γ has been shown to be a good indicator of anti-viral immunity in pigs, as well as in other species. In pigs, the measurement of IFN-γ levels has been found to be more useful for detecting the presence of antigen-specific, immunological memory than lymphoproliferative assays (Mateu de Antonio et al., 1998). The detection of antigen-specific IFN-γ production has been used to assess the cellular immunity of pigs in both pseudorabies (Zuckermann et al., 1998, 1999) and CSFV models (Suradhat et al., 2001; Suradhat and Damrongwatanapokin, 2003). In recent years, flow cytometric analysis has been extensively applied for studying the kinetics of cellular activation in various lymphocyte populations. The alpha-subunit of the high-affinity IL-2 receptor (CD25) is expressed on the surface of activated lymphocytes (Minami et al., 1993) and has been widely used to identify activated lymphocyte populations in various species including pigs (Dillender and Lunney, 1993; Quade and Roth, 1999; Piriou et al., 2003). However, the association between IFN-γ production and CD25 surface expression has never been studied in a pig model. To investigate the relationship between the two parameters, we developed an antigen-specific, intracellular cytokine (IFN-γ and interleukin-10, IL-10) staining assay and studied the kinetics of cytokine production in relation to CD25 expression by porcine lymphocyte subpopulations.

2. Materials and methods

2.1. Viruses

The CSFV reference strain, ALD strain, was a gift from the National Institute of Animal Health of Japan. The CSFV strain used for challenges was the Thai isolate (Bangkok 1950 strain) from the National Institute of Animal Health of Thailand (NIAH). Viruses were propagated in a SK-6 cell line. Infected cells were collected after 4 days incubation with a stock virus, and subjected to two freeze–thaw cycles. The viral suspension was centrifuged at $1000 \times g$ for 20 min. The collected supernatant is referred to as the stock virus. The stock viruses were kept at $-80\ ^{\circ}C$ until needed. Viral titers were determined by a peroxidase-linked virus titration assay using a previously described protocol (Pinyochon et al., 1999).

2.2. Monoclonal antibodies

The custom conjugated anti-swine CD8-PE-Cy5 conjugated mAb (76-2-11, IgG_{2a}) and anti-swine CD25-PE conjugated mAb (PGBL25A, IgG_1) were kindly provided by Dr. J.A. Roth (Iowa State University, Ames, USA). Anti-swine CD4-FITC conjugated mAb (74-12-4, IgG_{2b}), anti-CD4-PE conjugated mAb (74-12-4, IgG_{2b}), and biotinylated anti-swine IFN-γ mAb (P2C11, IgG_{2a}) were obtained from BD Biosciences (San Diego, CA, USA). The mAb anti-swine IL-10 (945A4C437B1, IgG_1) was obtained from Biosource International Inc. (Nivelles, Belgium). Strepavidin-FITC and goat–anti-mouse IgG_1-FITC conjugates were obtained from Serotec (Oxford, UK).

2.3. Animals and experimental protocols

Before the animal trials, the experimental and animal handling protocols were approved by the Ethics Committee on Experimental Animal Usage and Animal Welfare, Faculty of Veterinary Science, Chulalongkorn University.

2.3.1. Experiment 1: Cellular responses following vaccination with a CSF vaccine

Crossbred pigs, from sows that were routinely vaccinated with CSF vaccine, were maintained at the Faculty of Veterinary Science, Chulalongkorn University research farm in Nakorn Prathom province. The farm has no evidence of CSF outbreak in the last 2 years. Pigs were intramuscularly immunized twice (1 ml/dose) with a modified live, lapinized, Chinese strain, CSF vaccine (Department of Livestock Development, Thailand) at 5 and 7 weeks of age. Blood sampling was done every 2 weeks, from 3 to 13 weeks of age.

2.3.2. Experiment 2: Cellular responses following a CSFV challenge

Four-week-old, crossbred pigs from a CSF-free commercial farm were brought in and maintained at the animal facility, Faculty of Veterinary Science, Chulalongkorn University in Bangkok. Pigs were immunized intramuscularly with a modified live, lapinized, Chinese strain, CSF vaccine (1 ml/dose) (Department of Livestock Development, Thailand). The means of maternal derived serum-neutralizing titer of all groups were less than 1:8 at the time of vaccination. At 21 days post-vaccination (dpv), the pigs were challenged intramuscularly with 2×10^4 $TCID_{50}$ of the virulent CSFV, strain Bangkok 1950 (NIAH, Thailand). The control group was not vaccinated but received the same amount of the challenge virus. Naïve pigs were kept in a separate isolation unit throughout the experiment. Clinical signs were monitored daily and porcine peripheral blood mononuclear cell (PBMCs) were collected every 7 days for 2 weeks after the challenge.

2.4. Isolation of porcine PBMCs

Porcine peripheral blood mononuclear cells were isolated from 10 ml of the heparinized blood samples using Isoprep® separation medium (Robbins Scientific Cooperation, Sunnyvale, CA, USA) according to the manufacturer's protocol. The purified PBMCs were resuspended at a concentration of 6×10^6 cells/ml in RPMI 1640 (GIBCO, Carlsbad, CA, USA), supplemented with 10% calf serum (Starrate Ltd., Bethungra, NSW, Australia), 2 mM L-glutamine (GIBCO), 100 μM non-essential amino-acid (GIBCO), 1 mM sodium pyruvate (GIBCO), 50 μM 2-mercaptoethanol (Sigma Chemical Co., St. Louis, MO, USA) and 100 unit/ml of penicillin G, 100 μg/ml of streptomycin and 0.25 μg/ml of amphotericin B (antibiotic/antimycotic solution; GIBCO); this solution is referred as the complete medium.

2.5. Flow cytometry

2.5.1. In vitro activation and surface staining

For in vitro activation, 1 ml of freshly isolated PBMCs (6×10^6 cells/ml) were cultured in a well of 24-well plate, in the presence of CSFV (ALD strain) at one multiplicity of infection (m.o.i.) for 40 h. The cells were then harvested for triple-color fluorescent staining. The phenotypic analyses were done using the above mAbs (anti-CD4-FITC, anti-CD8-PE-Cy5, and anti-CD25-PE). Briefly, approximately 2×10^6 cells were washed with PBS supplemented with 0.5% bovine serum albumin (Sigma) and 0.1% sodium azide (Sigma) (FACS buffer), and distributed to the wells of 96-well plate. The cells were pelleted by centrifugation at $500 \times g$ for 2 min. The supernatants were discarded and the pellets were resuspended with

50 μl of the mixture of the three mAbs at appropriate concentrations, and incubated in the dark at 4 °C for 30 min. After incubation, the cells were washed three times with 150 μl/well of FACS buffer. After the final wash, the supernatants were removed and the cells were fixed by resuspending the pellet in 200 μl of 2% formaldehyde. Flow cytometric analyses were performed using a Facscan cytometer (BD Biosciences).

2.5.2. Intracellular cytokine-staining assay

Following in vitro activation with CSFV (see above) for 28 h, the protein transport inhibitor monensin (GolgiStop™, BD Biosciences) was added to the cell cultures, and the cells were incubated for another 12 h. In some experiments, PMA (50 ng/ml) and ionomycin (500 ng/ml) were added to the culture 3 h before harvesting. Cells were harvested and stained for surface expression of CD4 and CD8 using a mixture of anti-CD4-PE and anti-CD8-PE-Cy5 conjugates (see above), resuspended in FACS buffer at the appropriate concentrations. Following the last wash, the cells were fixed and permeabilized by resuspension in 200 μl/well of Cytofix/Cytoperm solution (BD Biosciences) and incubated for 3 h, in the dark at room temperature. Intracellular IFN-γ staining was performed using biotinylated anti-swine IFN-γ mAb followed by a strepavidin-FITC conjugate resuspended in BD Perm/Wash solution provided with the BD Cytofix/Cytoperm kit (BD Bioscience). Intracellular IL-10 staining was performed using a mAb anti-swine IL-10 followed by FITC-conjugated, goat–anti-mouse IgG_1antibody. All of the washing processes following permeabilization were done using the BD Perm/Wash solution, according to the manufacturer's protocol. At the final step, the cells were resuspended in 200 μl of 2% formaldehyde and kept in the dark at 4 °C until needed. Flow cytometric analysis was performed using a Facscan cytometer (BD Biosciences).

2.6. Flow cytometric analyses

The viability of studied population prior to surface staining, determined by Trypan blue staining, was more than 80%. Numbers of gate events for analyses of surface markers and intracellular cytokines were 10,000 and 100,000, respectively. Lymphocytes were first gated (G1) from the harvested population by size (FSC) and granularity (SSC), this yielded predominantly lymphocyte population with approximately 3% $SWC3^+$ cell contamination (data not shown). The numbers of lymphocyte subpopulations ($CD4^+CD8^-$, $CD4^-CD8^+$, $CD4^-CD8^-$, and $CD4^+CD8^+$) were determined by the percentages of CD4 and/or CD8 positive cells from G1. For analyses of triple labeled cells, the G2 ($CD8^+$) and G3 ($CD4^+$) were selected from the lymphocyte (G1) population and used for determining of the expression of the second (CD4 or CD8) and third parameter (CD25 or cytokine). Since

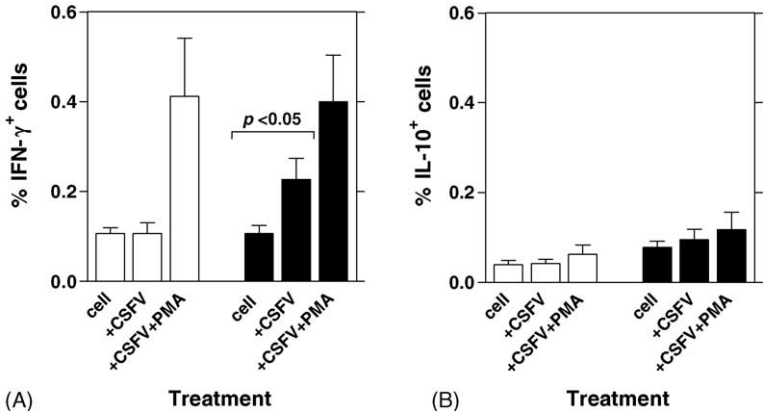

Fig. 1. Levels of IFN-γ (A) and IL-10 (B) positive cells in lymphocyte population from naïve pigs (white bar) and CSFV-primed pigs (black bar). Porcine PBMCs were in vitro cultured alone (cell), with CSFV for 40 h (+CSFV), or with CSFV for 40 h and an addition of PMA and ionomycin 3 h prior to cell harvesting (+CSFV + PMA). The data represent the mean ± S.E.M. of the percentage positive cells from five animals.

porcine $CD8^+$ population exists in $CD8^{hi}$ and $CD8^{lo}$ forms (Zuckermann, 1999), the triple positive, $CD4^+CD8^+CD25$ (or cytokine)$^+$, population were determined from G3.

2.7. Statistical analysis

All statistical analyses were performed using GraphPad Prism® Version 3.00 for Windows (GraphPad Software, San Diego, CA, USA). Either *t*-test or one-way ANOVA, followed by a post-test (Tukey's multiple comparison) when significant differences at the 0.05% confident level were present, was used as indicated.

3. Results

3.1. Interferon-γ production of by porcine lymphocytes in response to the recall antigen

Previously, porcine PBMCs have been shown to produce IFN-γ in response to the recall antigen (classical swine fever virus) by an ELISPOT assay (Suradhat et al., 2001). In this study, using the same in vitro activation system, we established an intracellular cytokine-staining assay to further characterize the CSFV-specific cytokine producing cells. The levels of cytokine production by porcine PBMCs, in response to in vitro CSFV exposure, were determined before vaccination (5 weeks old) and 2 weeks after the second immunization (9 weeks old) (see Section 2.3.1). Our result showed that the CSFV-primed lymphocytes could produce IFN-γ in response to in vitro exposure with CSFV ($p < 0.05$, *t*-test). In addition, the level of IFN-γ producing cells from the vaccinated group was significantly higher ($p < 0.05$, *t*-test) than that from the naïve group (Fig. 1A). Although lymphocytes from the naïve group did not show enhanced IFN-γ production when cultured with CSFV, the lymphocytes from both naïve and vaccinated groups produced comparable level of IFN-γ^+ cells when stimulated with PMA and ionomycin (PMA/I). This result indicated that addition of CSFV into the culture system did not affect the ability to produce IFN-γ by the lymphocytes of the naïve pigs (Fig. 1A). In the same study, addition of CSFV or CSFV and PMA/I did not significantly affect the level of IL-10 production by porcine lymphocytes from both groups (Fig. 1B).

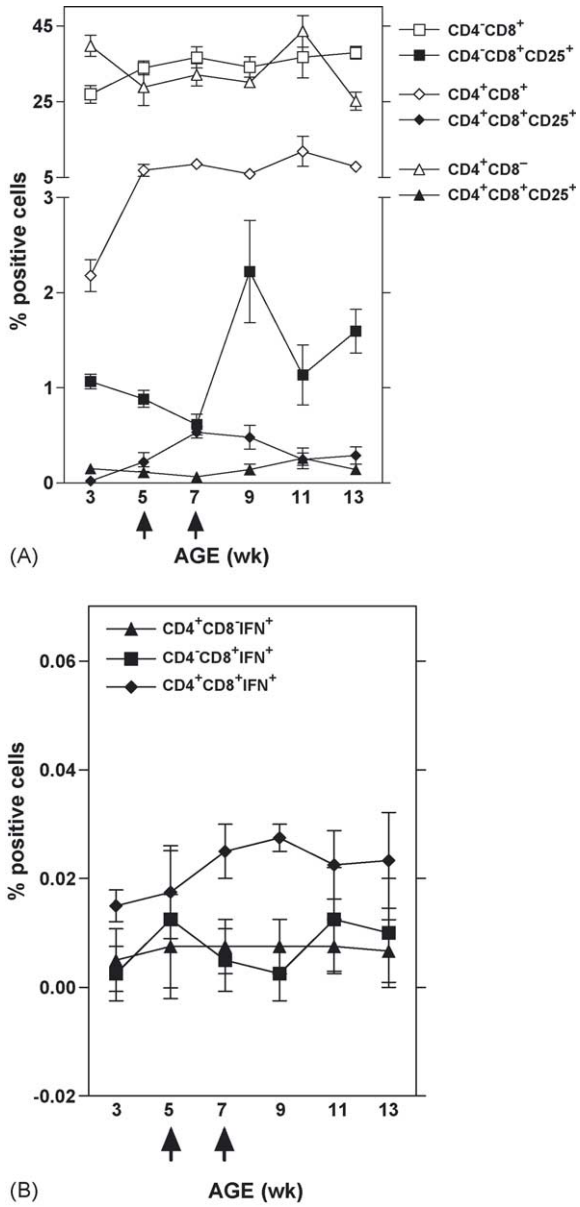

Fig. 2. Kinetics of CD25 surface expression (A), and intracellular IFN-γ production (B) in lymphocyte subsets, in PBMCs isolated from pigs vaccinated twice with CSF vaccines at 5 and 7 weeks (arrow). Porcine PBMCs were cultured in vitro with CSFV for 40 h before flow cytometric analyses. The data represent the mean ± S.E.M. of the percentage positive cells from four animals.

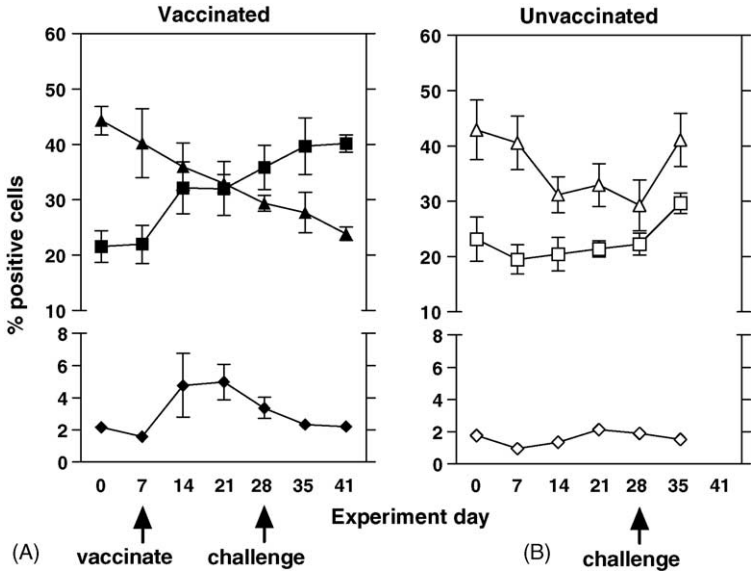

Fig. 3. Percentages of $CD4^+CD8^-$ (triangle), $CD4^-CD8^+$ (square) and $CD4^+CD8^+$ (diamond) cells from PBMCs of the vaccinated (A) and unvaccinated pigs (B). Pigs were immunized on day 7 with CSF vaccine and challenged with the virulent CSFV on at 21 dpv (day 28). Porcine PBMCs were cultured in vitro with CSFV for 40 h before flow cytometric analyses. The data represent the mean ± S.E.M. of the percentage positive cells from four animals.

3.2. Kinetics of CD25 expression and IFN-γ production by porcine PBMCs in vaccinated pigs

To investigate the relationships between CSFV-specific IFN-γ production and the expression of the IL-2R (CD25) by porcine PBMCs following vaccination, the two parameters were monitored from the same pigs at the age of 3–13 weeks old (see Section 2.3.1). Following vaccination, there was a slight increase in the activated memory lymphocyte subpopulation ($CD4^+CD8^+CD25^+$ cells), which diminished within a few weeks. The number of activated cytotoxic T cells ($CD4^-CD8^+CD25^+$ cells) peaked approximately 2 weeks after the memory population. Most of the activated lymphocyte population detected in PBMCs following in vitro activation carried the $CD4^-CD8^+$ phenotype. It should be noted that total percentage of $CD4^-CD8^+$ T cells in the PBMCs remained relatively stable throughout the experiment. Thus, it is unlikely that the increased number of $CD4^-CD8^+CD25^+$ T cells was due to a relative increase in the $CD4^-CD8^+$ T cell subpopulation (Fig. 2A). In contrast to the CD25 expression, different T cell subpopulation was found responsible for the IFN-γ production observed in the PBMCs following CSFV vaccination. The result showed that $CD4^+CD8^+$ T cells were the major IFN-γ producers in PBMCs throughout the experiment, while the number of IFN-γ

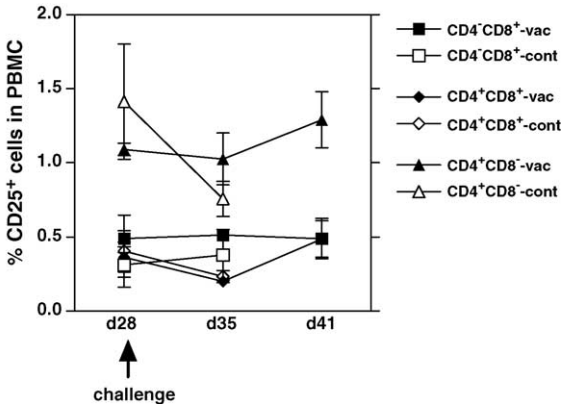

Fig. 4. Kinetics of CD25 surface expression on lymphocyte subsets in the PBMCs of pigs vaccinated with CSF vaccines and challenged with virulent CSFV at 21 dpv (day 28). Porcine PBMCs were cultured in vitro with CSFV for 40 h before flow cytometric analyses. The data represent the mean ± S.E.M. of the percentage positive cells from four animals.

producing $CD4^{-}CD8^{+}$ cells was relatively low and comparable to that of the IFN-γ producing $CD4^{+}CD8^{-}$ cells (Fig. 2B). In addition, the kinetic of IFN-γ production was resembled to that of activated $CD4^{+}CD8^{+}$ T cells. In this experiment, cellular activation and IFN-γ production in the naive $CD4^{+}CD8^{-}$ population following vaccination was minimal (Fig. 2).

3.3. Kinetics of CD25 expression and cytokine production by porcine PBMCs following CSFV challenge

To determine the kinetics of CD25 expression and cytokine production following the CSFV challenge, crossbred pigs (4 pigs/group) were immunized once with a CSF vaccine and challenged with a virulent

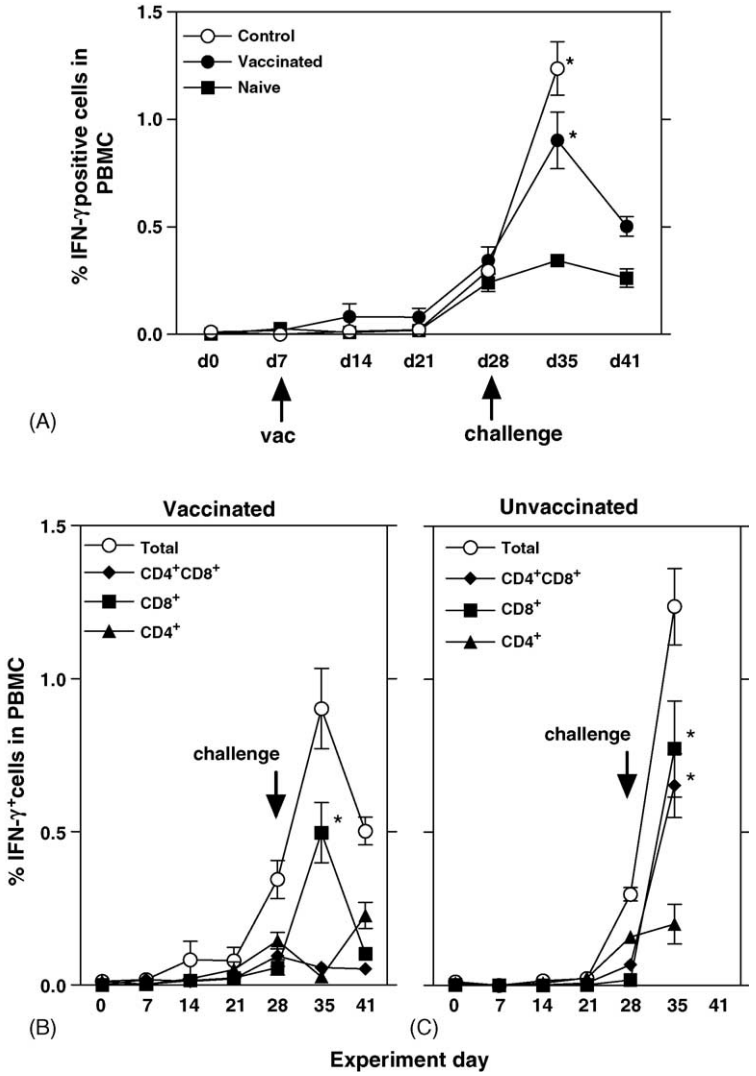

Fig. 5. Kinetics of total IFN-γ production by porcine lymphocytes (A) and by lymphocyte subsets from the PBMCs of vaccinated (B) and unvaccinated (C) group. Pigs were vaccinated with CSF vaccines (day 7) and challenged with virulent CSFV at 21 dpv (day 28). Porcine PBMCs were cultured in vitro with CSFV for 40 h before flow cytometric analyses. The studied lymphocyte subsets included $CD4^{+}CD8^{-}$ ($CD4^{+}$), $CD4^{-}CD8^{+}$ ($CD8^{+}$) and $CD4^{+}CD8^{+}$ populations. The data represent the mean ± S.E.M. of the percentage positive cells from four animals. The symbol (*) indicates significant difference from the data of day 28 ($p < 0.05$, paired t-test).

strain of CSFV (Bangkok 1950) at 21 dpv (Section 2.3.2). Unvaccinated pigs also received the same amount of the challenge virus at the same time. Following the CSFV challenge, the unvaccinated pigs exhibited severe clinical signs of CSF starting from 7 days post-infection (dpi). All of the unvaccinated pigs developed severe leukopenia, with insufficient cells to analyze after the first week of infection, and died within 14 days following the challenge. Therefore, there was no data on the lymphocyte subpopulation from the control pigs after day 35. All of the vaccinated pigs and naïve pigs remained clinically normal through the end of the experiment.

Following vaccination, the numbers of $CD4^{-}CD8^{+}$ and $CD4^{+}CD8^{+}$ from vaccinated group slightly increased, compared to those from the control group (Fig. 3). However, due to variation of the lymphocyte numbers from pigs within the same group, the changes in the lymphocyte numbers between days 7 and 14 were not statistically significant. Nevertheless, the number of $CD4^{-}CD8^{+}$ cells of the vaccinated group gradually increased, while the number of $CD4^{+}CD8^{-}$

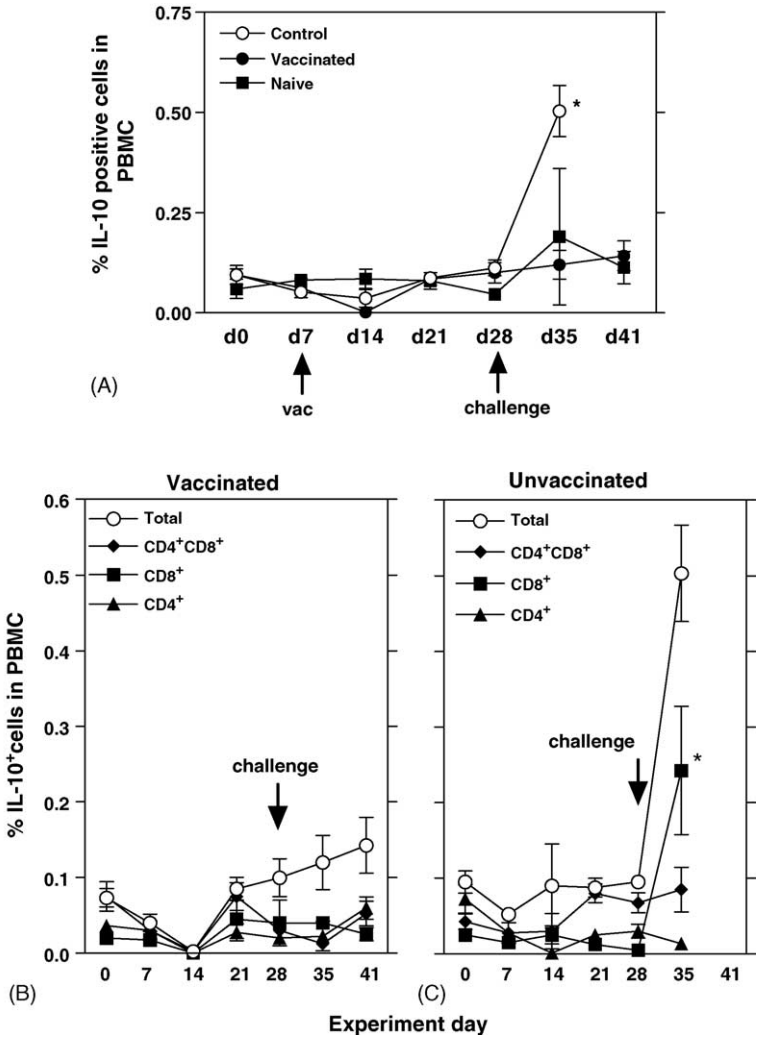

Fig. 6. Kinetics of total IL-10 production by porcine lymphocytes (A) and by lymphocyte subsets from the PBMCs of vaccinated (B) and unvaccinated (C) group (see Fig. 5). The symbol (*) in part (A) indicates significant difference from other groups ($p < 0.05$, ANOVA followed by Tukey's multiple comparison test). The symbol (*) in part (C) indicates significant difference from the data of day 28 ($p < 0.05$, paired t-test).

cells decreased, after vaccination through the end of the experiment (Fig. 3A). Numbers of the studied lymphocyte subpopulation of the control group remained relatively unchanged throughout the experiment (Fig. 3B).

At the time of challenge (day 28), no significant differences in the numbers of $CD25^+$ and IFN-γ^+ cells among the groups were observed (Figs. 4 and 5). Interestingly, the numbers of $CD25^+$ cells among the studied populations also remained unchanged following the challenge (Fig. 4). In contrast to the pattern of CD25 expression, there were significant increases in the numbers of IFN-γ producing cells in both vaccinated and unvaccinated groups following challenge (Fig. 5A). Exposure to virulent CSFV significantly increased the number of IFN-γ producing cells in the PBMCs of both vaccinated ($p < 0.05$, paired *t*-test) and unvaccinated pigs ($p < 0.01$, paired *t*-test) at 7 dpi. The numbers of IFN-γ producing cells in the vaccinated group fluctuated after challenge. At day 35, the $CD4^-CD8^+$ population was major IFN-γ producing cells in vaccinated pigs, while both $CD4^+CD8^+$ and $CD4^-CD8^+$ populations contributed to the IFN-γ production in the unvaccinated group (Fig. 5B and C). Although, the number of IFN-γ producing cells in the unvaccinated group markedly increased by 7 dpi, these pigs did not survive the challenge. Interestingly, the number of IL-10 producing cells in the unvaccinated group also significantly increased and higher than the other groups at 7 dpi ($p < 0.05$, ANOVA followed by Tukey's multiple comparison test) (Fig. 6A). The majority of the IL-10 producing cells were the $CD4^-CD8^+$ population (Fig. 6C). No significant changes in IL-10 production were observed in the vaccinated group following the challenge (Fig. 6A).

4. Discussion

Like other species, pigs have typical $CD4^+CD8^-$ and $CD4^-CD8^+$ T lymphocytes in their peripheral blood and lymphoid tissues. These cells have been shown to have helper and cytolytic functions, respectively. However, unlike humans and mice, pigs also have an extrathymic double positive lymphocyte population that comprises 8–64% of the circulating pool of small resting T cells (reviewed in Zuckermann, 1999). It was recently shown that the extrathymic naïve Th cells ($CD4^+CD8^-$) can upregulate their CD8 expression upon cellular activation. Therefore, by examining the expression of the CD4 and CD8 molecules, porcine Th populations can be categorized into resting Th cells ($CD4^+CD8^-CD25^-$), activated Th cells ($CD4^+CD8^-CD25^+$) and memory (or primed) Th cells ($CD4^+CD8^+CD25^-$) (Saalmuller et al., 2002). In this experiment, we followed the number of single positive and double positive porcine lymphocyte subsets in PBMCs following exposure to CSF antigen. We also monitored the expression of the activation marker (CD25) and intracellular IFN-γ production in these subpopulations. It should be noted that the PBMCs were cultured in vitro with CSFV before flow cytometric analyses. Therefore, kinetics of cellular activation and cytokine production obtained from this experiment would reflect CSFV-specific responses in the PBMCs.

It has been shown in several studies that the level of IFN-γ production can be used as an indicator for cell-mediated immunity in pigs (Mateu de Antonio et al., 1998; Zuckermann et al., 1998; Suradhat et al., 2001). Our result from this study clearly showed that primed porcine PBMCs produced IFN-γ, but not IL-10, in response to secondary exposure to CSFV, and that the response could be measured by flow cytometry (Fig. 1). Following vaccination with CSF vaccine, double-positive (DP), $CD4^+CD8^+$ cells were the major IFN-γ producers in response to in vitro activation (Fig. 2). This finding is in agreement with previous reports showing that the DP cells are a memory population, which can produce high levels of IFN-γ in response to a recall antigen or polyclonal activator (Rodriguez-Carreno et al., 2002; Saalmuller et al., 2002). The finding also implies that that the IFN-γ producing cells detected by ELISPOT assays following immunization with a CSF vaccine (Suradhat et al., 2001; Suradhat and Damrongwatanapokin, 2003) were indeed reflecting helper T lymphocyte (Th) activity. It should also be noted that the number of IFN-γ producing cells following vaccination was not as high as that observed following the viral challenge. This finding is consistent with a previous report showing that immunization with a modified live vaccine induces a lower level of cell-mediated response than infection (Piriou et al., 2003). In Experiment 2, the vaccinated group did not show any

significant increase in the number of IFN-γ producing cells following vaccination, compared to a sharp increase in the numbers of IFN-γ producing cells following the CSFV challenge (Fig. 5). The indifferences in cytokine production among the groups following vaccination might be partly related to the low levels of detectable IFN-γ producing cells, and the high variation of the numbers of cytokine producing cells from pigs within the same group. In our experience, detection of IFN-γ producing cells following CSF vaccination by flow cytometry is not always as sensitive as the previously reported ELISPOT assay. It should be noted that on day 28 of Experiment 2, the numbers of IFN-γ producing cells in all groups increased considerably without any specific treatment (Fig. 5A). The reason for this fluctuation is no clearly known, but could relate to non-specific activation of the lymphocytes, as there seemed to be increased activity on the cytokine production of the naïve $CD4^+$ cell (Fig. 5B and C).

Following vaccination for CSF, there seemed to be two phases of cellular activation measured by the upregulation of surface CD25 expression; early cellular activation of the DP population is followed by the activation of the $CD4^-CD8^+$ T lymphocytes (Fig. 2A). Although the CD25 marker has been used to identify cellular activation in several reports (Dillender and Lunney, 1993; Quade and Roth, 1999; Saalmuller et al., 2002; Piriou et al., 2003), however, we did not see a good correlation between the level of CD25 expression and cytokine production in PBMCs either following vaccination or after challenge (Figs. 2 and 4). In this study, the activated population might not always be the major population producing the cytokine in response to the CSFV antigen. The differences in dynamics of CD25 expression and cytokine production might be related to the specific lymphocyte population in each study. Unlike the cytokine production, which is shown to be antigen-specific (Fig. 1), combining the percentages of $CD25^+$ cells from the three studied lymphocyte subpopulations ($CD4^+$, $CD8^+$, and double positive) contributed to less than 50% of the $CD25^+$ cells from total lymphocyte population, in both naïve and primed populations (data not shown). In addition, no significant differences in the numbers of $CD25^+$ cells were observed in the challenge pigs regardless of their immunological history (Fig. 4). These results suggested that lymphocytes could also be activated, non-specifically, and upregulated CD25 expression by CSFV. Alternatively, it is also possible that the incubation period for the in vitro activation system used in this study is shorter than in previous reports (Saalmuller et al., 2002; Piriou et al., 2003). Therefore, the results could reflect the different dynamics of cellular activation at a different incubation time. Development of the four-color staining assay that simultaneously analyses CD25 expression and IFN-γ production, in addition to the surface markers, will provide a better explanation on this discrepancy.

Following challenge with CSFV, the number of IFN-γ producing cells in the PBMC population increased in both vaccinated and unvaccinated pigs at 7 dpi (Fig. 5). Interestingly, the $CD4^-CD8^+$ population was the subpopulation mainly responsible for IFN-γ production in the PBMCs of both groups following challenge, while there were a significantly less number of IFN-γ producing DP cells in the PBMCs of the vaccinated group during the first week of infection (Fig. 5A). This finding is in agreement with the previous report demonstrating that the activation of $CD8^+$ CTLs was observed early (from 15 dpi) in the PBMCs of immunized pigs challenged with CSFV, but the activation of $CD4^+CD8^+$ cells was not observed until 35 dpi (Piriou et al., 2003). As it has been previously shown that the porcine memory population preferentially home to the secondary lymphoid organs (Zuckermann, 1999). The low number of antigen-specific DP cells detected in the PBMCs of primed pigs following the CSFV challenge could simply reflect the difference in tissue homing preference among the subpopulations during an effector phase of the immune response.

It should be noted that porcine gamma–delta (γδ) T lymphocytes can also expressed the CD8 surface molecules (Pescovitz, 1998). However, the proportion of γδ T lymphocytes in the PBMCs of young pigs is usually low and they are not the major IFN-γ producers in the PBMCs even when stimulated with a polyclonal T cell activator (Rodriguez-Carreno et al., 2002). In addition, γδ T cells are more sensitive to CSFV infection and are depleted early after the viral challenge (Pauly et al., 1998). Although the kinetics of CD25 expression and IFN-γ production by γδ T lymphocytes were not monitored in this study, it is unlikely that the γδ T cells be the major IFN-γ

producers observed in the $CD4^{-}CD8^{+}$ population following the viral challenge.

Although the number of IFN-γ positive cells was markedly increased in the unvaccinated pigs at 7 dpi (Figs. 3 and 4), these pigs did not survive the CSFV challenge. This phenomenon, which is usually seen in pigs with overwhelming CSFV infections, has been previously demonstrated using an ELISPOT assay (Suradhat et al., 2001). Interestingly, the number of IL-10 producing cells in the PBMCs was also markedly increased in the unvaccinated pigs, while the level of IL-10 production in the vaccinated pigs remained low through the end of the experiment (Fig. 6). The $CD8^{+}$ cells were also found the major IL-10 producer in PBMCs (Fig. 6C). Thus, our results demonstrated that CSFV infection significantly increased the levels of both IFN-γ and IL-10 production in unvaccinated pigs. It should be noted that although CSFV is not usually detected in the PBMC population during the first week of infection, but abrogation of cellular immune responses is observed as early as 5 dpi (Pauly et al., 1998). Interleukin-10 is widely accepted to be a potent immunosuppressive cytokine that can strongly inhibit both innate and specific immune functions (Moore et al., 2001; Redpath et al., 2001). Our findings suggest that cytokine dysregulation could be one of the underlying mechanisms resulting in the immunoinhibitory effect observed following CSFV infection. Recently, the immunoinhibitory effects of viral infection via IL-10 induction have also been observed in hepatitis C virus (Dolganiuc et al., 2003) and human immunodeficiency virus (Almonti et al., 2003) models.

Taken together, our results suggest that CD25 expression and antigen-specific IFN-γ production by porcine lymphocytes may not be tightly associated. Furthermore, different lymphocyte populations appear to be responsible for cytokine production in the PBMCs following CSF vaccination and challenge. Further understanding of the kinetics of the cellular response against CSFV may provide information about the mechanisms of protection and a better strategy for disease control in the future.

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