และ IL-2 gene นั้นไม่มีเปลี่ยนแปลงมากนักในระบบการศึกษานี้ ซึ่งอาจแตกต่างจากผลการศึกษาที่ได้จากสัตว์ต่าง species แต่อย่างไรก็ตามก็เคยมีการตั้งข้อสังเกตจากคณะผู้วิจัยอื่นที่ศึกษาการสร้าง mRNA ของ gene ดังกล่าวในสุกร โดยใช้เทคนิค quantitative real-time PCR และไม่สามารถตรวจพบการสร้าง IL-2 และ IL4 mRNA ได้จากเซลล์ที่ทำการกระตุ้นด้วย mitogen หลายชนิดได้เช่นกัน ซึ่งผลการทดลองนี้ชี้ว่าสุกรอาจมีวิธีการใช้ cytokine ทั้ง 2 ชนิด ที่แตกต่างจากที่มีรายงานในมนุษย์และหนูทดลอง (Reddy et al., 2000)

ในการศึกษาครั้งนี้ ผู้วิจัยพบว่า PRRSV มีผลในการกระตุ้นการแสดงออกของ IL-10 gene อย่างชัดเจนในทั้ง 2 การทดลอง ผลการศึกษานี้ยังไม่เคยมีผู้รายงานมาก่อน แม้ว่าผลจากการศึกษาในครั้งนี้จะเป็นการศึกษาในระยะต้นและไม่ได้มีการตรวจวัดระดับของ IL-10 ที่สร้างขึ้นจริง แต่จากการวิเคราะห์ข้อมูลที่ผ่านมาพบว่าการสร้าง IL-10 ในตัวสัตว์ที่ติดเชื้อค่อนข้างจะสอดคล้องกับปรากฏการณ์หลายๆ อย่างที่เกิดขึ้นในระหว่างการติดเชื้อ PRRSV ดังจะอภิปรายต่อไป นอกจากนี้ยังเคยมีการรายงานมาก่อนว่าในลูกสุกรที่เกิดจากแม่ที่เคยได้รับเชื้อ PRRSV มาก่อนจะสามารถตรวจพบการสร้าง IL-10 จาก PBMC ได้ในปริมาณสูงได้เช่นกัน (Feng et al., 2000)

เป็นที่ยอมรับกันโดยทั่วไปว่า IL-10 เป็น cytokine ที่มีฤทธิ์กดการทำงานของระบบภูมิคุ้มกัน การใช้ IL-10 เพื่อให้เกิดประโยชน์ในการเพิ่มความสามารถในการดำรงชีวิตอยู่ใน host ถือเป็นวิธีการหนึ่งที่มีการนำมาใช้อยู่บ่อยครั้งโดยเชื้อที่อาศัยอยู่ภายในเซลล์ โดยเฉพาะอย่างยิ่งเซลล์ในกลุ่ม monocyte/macrphage เชื้อโรคบางชนิดกระตุ้นให้ host สร้าง IL-10 ในขณะที่เชื้อโรคอีกหลายชนิดได้พัฒนาความสามารถในการสร้าง IL-10 ด้วยตัวเอง (Redpath et al., 2001; Fickenscher et al., 2002) IL-10 มีฤทธิ์กดการทำงานของเซลล์ในระบบภูมิคุ้มกันหลายชนิดตั้งแต่ monocyte macrophage dendritic cell NK-cell รวมไปถึง T cell โดยเฉพาะอย่างยิ่ง Th1 cell (Moore et al., 2001) IL-10 มีฤทธิ์กดการสร้าง proinflammatory cytokine หลายชนิด จากเซลล์กลุ่ม monocyte/macrophage รวมทั้ง tumor necrosis factor- alpha (TNF-α) ซึ่งจัดเป็น cytokine ที่สำคัญที่สุดตัวหนึ่งในการเริ่มต้น cytokine cascade ของขบวนการอักเสบ (Abbas et al., 2000) เป็นที่น่าสังเกตว่าหลังการติดเชื้อ PRRSV จะแทบไม่สามารถตรวจวัดการสร้าง TNF-α ได้เลยจากเซลล์ที่แยกได้จากปอด (Van Reeth et al., 1999) ทั้งยังพบการกดการสร้าง IFN-α อย่างชัดเจนโดยไม่ได้มีสาเหตุเกี่ยวข้องกับการตายของเซลล์ (Albina et al., 1998) และนอกจากนี้ยังเคยมีการรายงานไว้แล้วว่า PRRSV สามารถกดการทำงานของเซลล์ในกลุ่ม monocyte/macrophage ทั้งในด้าน phagocytic และ bacteriocidal activity (Thanawongnuwech et al., 1998) ซึ่งจะมีผลลดความสามารถของเซลล์เหล่านี้ในการกำจัดเชื้อออกจาก host อีกด้วย ข้อมูลเหล่านี้แสดงให้เห็นถึงการกดการทำงานของเซลล์ในกลุ่ม monocyte และ macrophage โดยเชื้อ PRRSV อย่างชัดเจน ซึ่งสอดคล้องกับลักษณะทางพยาธิสภาพที่พบในปอดของสัตว์ที่ติดเชื้อ PRRSV ที่มักจะไม่พบการอักเสบของเนื้อเยื่อมากนักเมื่อเทียบกับไวรัสชนิดอื่น แต่ในขณะเดียวกันก็มีการเรียก target cell เหล่านี้เข้ามาในบริเวณเพิ่มมากขึ้น (Van Reeth and Nauwynck, 2000) การกดการทำงานของเซลล์เหล่านี้ยังอาจสามารถนำไปใช้อธิบายการติดเชื้อแทรกซ้อนที่ปอดภายหลังการติดเชื้อ PRRSV ที่มักพบได้อยู่เสมอในสภาพการเลี้ยงจริง นอกจากนี้การกดการทำหน้าที่ของเซลล์เหล่านี้ในแง่ของการเป็น antigen-presenting cell จะส่งผลให้มีความล่าช้าของการกระตุ้นการทำงานของ specific immunity และเปิดโอกาสให้เชื้อ PRRSV จึงมีโอกาสเพิ่มจำนวนในร่างกายอย่างมีประสิทธิภาพ ซึ่งตรงกับลักษณะของการตอบสนองทางภูมิคุ้มกันภายหลังการติดเชื้อ PRRSV ที่มักจะช้ากว่าที่ควรจะเป็น ดังนั้นเมื่อพิจารณาจากความจำเพาะของเซลล์ที่ PRRSV สามารถเจริญอยู่ได้ และลักษณะของพยาธิสภาพ และการตอบสนองทางภูมิคุ้ม จึงเป็นไปได้ว่า PRRSV จะพัฒนาความสามารถในการกระตุ้นการสร้าง IL-10 ภายใน host เพื่อประโยชน์ของตัวไวรัสเอง

ผลจากการศึกษาในครั้งนี้ยังชี้ให้เห็นว่าการมีการติดเชื้อ PRRSV ร่วมด้วยอาจส่งผลรบกวนการตอบสนองทางภูมิคุ้มต่อ antigen ชนิดอื่นอีกด้วย (การศึกษาที่ 2) แม้ว่าการศึกษานี้จะไม่ได้ลงลึกไปถึงกลไกของการรบกวน แต่ก็อาจใช้อธิบายถึงปรากฏการณ์ที่เกิดขึ้นในสภาวะการเลี้ยงจริง ซึ่งมักพบว่าการติดเชื้อ PRRSV มักจะก่อให้

เกิดการรบกวนประสิทธิภาพของวัคซีนชนิดอื่นที่ใช้กลไกของเซลล์ในการกำจัดเชื้อพิษ (De Bruin et al., 2000; Thacker et al., 2000)

แม้ว่าการศึกษานี้ได้ดำเนินมาสู่ระยะสุดท้ายของโครงการ ผลที่ได้รับจากงานวิจัยทำให้สามารถพัฒนาเทคนิคสำหรับการศึกษาการตอบสนองทางภูมิคุ้มกันชนิดเซลล์โดยการตรวจวัดระดับการแสดงออกของ cytokine gene ชนิดต่างๆ และชี้ให้เห็นอิทธิพลของเชื้อ PRRSV ต่อการแสดงออกของ cytokine gene ซึ่งถือเป็นองค์ความรู้ใหม่ที่ยังไม่เคยมีผู้ศึกษาอย่างจริงจังมาก่อน อย่างไรก็ตามผู้วิจัยเชื่อว่าผลจากงานวิจัยนี้จะเป็นจุดเริ่มของการศึกษาในแนวลึกอีกมากมาย เพื่อเพิ่มความเข้าใจในพยาธิกำเนิดของโรค PRRS ให้ดีขึ้น และจะเป็นข้อมูลสำคัญที่จะใช้ในการวางแนวทางในการจัดการและป้องกันการเกิดโรคในสุกรที่มีประสิทธิภาพและเป็นผลประโยชน์โดยตรงแก่เกษตรกรในประเทศต่อไป นอกจากนี้ผู้วิจัยเชื่อมั่นว่าเทคนิคที่พัฒนาขึ้นนี้จะสามารถนำไปประยุกต์ในในการศึกษาที่เกี่ยวข้องกับปัจจัยที่มีผลต่อการทำงานของระบบภูมิคุ้มกันในสุกรได้ในอีกหลายๆ ทาง

Output ที่ได้จากโครงการ

1. **Poster presentation**

   Federation of Immunological Societies of Asia-Oceania (FIMSA) advanced course and conference; "Molecular mechanisms of infection and immunity" 21-25 October 2002, Ayutthaya Thailand.

   Effect of porcine reproductive and respiratory syndrome virus on porcine cytokine gene expression

   Suradhat, S, Thanawongnuwech, R., and Poovorawan, Y.

2. **Manuscript** (คาดว่าจะส่งตีพิมพ์ใน Journal of General Virology หรือ Viral Immunology)

   Upregulation of IL-10 gene expression in porcine peripheral blood mononuclear cells by Porcine Reproductive and Respiratory Syndrome Virus

   Suradhat, S., Thanawongnuwech, R., and Poovorawan, Y.

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ภาคผนวก

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EFFECT OF PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS ON THE PORCINE CYTOKINE GENE EXPRESSION

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To study the role of Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) on porcine cytokine production, we established the multiplex PCR assay, which allowed semi-quantitative analysis of porcine cytokine, IFN-γ, IL-2, IL-4, and IL-10 gene expression simultaneously from porcine peripheral blood mononuclear cells (PBMC). In the presence of PRRSV, the IL-10 gene expression in porcine PBMC, isolated from naïve pigs, was prominently upregulated. In another separate experiment, in which PBMC from pigs, previously primed with a classical swine fever virus (CSFV) vaccine, were used for *in vitro* stimulation, the PBMC cultured in the presence of the recall antigen, CSFV, exhibited an enhanced IFN-γ gene expression. However, a significant reduction of IFN-γ and upregulation of IL-10 gene expression were observed in the PBMC cultured in the presence of CSFV and PRRSV. This finding indicated that the presence of PRRSV enhanced the IL-10 gene expression in porcine PBMC, and could significantly interfere with the recall antigen response. In both experiments, the changes in IL-2 and IL-4 gene expression were minimal. Our results implied that enhanced IL-10 production might be one of the strategies used by PRRSV to regulate the host immune system.

2. Manuscript (submitted to The Journal of General Virology)

Upregulation of IL-10 gene expression in porcine peripheral blood mononuclear cells by Porcine Reproductive and Respiratory Syndrome Virus (PRRSV)

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Summary

Several lines of evidence suggest that Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) may have immunomodulatory effects on the host immune system. To study the role of PRRSV on porcine cytokine production, we established the multiplex PCR that allowed a semi-quantitative analysis of IFN-γ, IL-2, IL-4, IL-10 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene expression from porcine peripheral blood mononuclear cells (PBMC). Our results showed that PRRSV predominantly upregulated the IL-10 gene expression in porcine PBMC. When PBMC isolated from pigs, previously primed with a classical swine fever virus (CSFV) vaccine, were cultured with CSFV and PRRSV, significant upregulation of IL-10 gene expression and reduction of IFN-γ gene expression were observed, indicating that the presence of PRRSV had influence on the recall antigen response. Our results implied that the enhanced IL-10 production might be one of the strategies used by PRRSV to modulate the host immune system.

Introduction

Porcine Reproductive and Respiratory Syndrome (PRRS) is caused by Porcine Reproductive and Respiratory Syndrome Virus (PRRSV), an enveloped positive-stranded RNA virus that belongs to the family *Arteriviridae* (Snijder & Meulenberg, 1998). PRRSV has been recognized as one of the major etiological agents of Porcine Respiratory disease Complex (PRDC) which causes a serious health problem in pig industry worldwide (Halbur, 1998). Although the mechanism(s) by which PRRSV undertakes to invade the host immune system is unclear, several lines of evidence imply that PRRSV may negatively modulate the host immune system.

PRRSV are phenotypically highly variable, and generally cause a persistent infection with a wide range of secondary infection (Wardley *et al.*, 1996). Following initial infection, PRRSV persists in infected pigs up to 12 weeks and the infected pigs can shed infectious virus (Will *et al.*, 1997). Although PRRSV is highly contagious, the virus replication appears to be limited mainly within the phagocytic cell populations including macrophages and monocytes. This population was known to be the major effector cells of the lungs. Interestingly, in most cases, there is a lack of correlation between the amount of viral antigen and the degree of pathological lesions. These findings imply a possibility of immune mediated pathogenesis rather than the direct effect from viral infection (reviewed in Lager & Mengeling, 2000). In addition, several proinflammatory cytokines were undetectable or minimally increased following the exposure to PRRSV, as compared to other respiratory viruses (Van Reeth & Nauwynck, 2000).

The immune responses to PRRSV have been studied extensively, and viral-specific cellular responses in pigs have been demonstrated, including lymphocyte proliferation, delayed type hypersensitivity response, cytotoxic activity, and cytokine production. However, there seemed to be a delay in the responses upon PRRSV infection, as compared to other viral infections. Cellular response to PRRSV is not usually detected until 4 weeks following PRRSV infection (Bautista & Molitor, 1997; Lopez Fuertes *et al.*, 1999), whereas the cellular immune response to other virus, for example classical swine fever virus (CSFV), can be detected within a week following viral infection (Suradhat *et al.*, 2001). In addition, although PRRSV induces a strong antibody response within the first week post infection, neutralizing antibodies are only detected at 4 weeks post-infection, long after the virus is cleared from the blood circulation (Yoon *et al.*, 1995). Thus, there appears to be a delay in induction of both arms of immune responses in PRRSV infected pigs.

Cytokines play crucial role in induction and regulation of immune responses. However, studies of the role of cytokine in immune regulation in pigs have been limited due to the lack of porcine cytokine-specific immunological and biological assays. Recently, knowledge on the role of cytokines for immunopathology and host-pathogen interactions has been increased rapidly (Wood & Seow, 1996). A reverse-transcription-polymerase chain reaction (RT-PCR) has been shown to be a sensitive and effective method for measuring cytokine mRNA expression in porcine samples (Dozois *et al.*, 1997; Reddy *et al.*, 2000; Thanawongnuwech *et al.*, 2001). The relative levels of cytokine expression can be semi-quantitatively analyzed by normalizing the target mRNA with the reference (i.e. housekeeping gene) transcripts. Multiplex polymerase chain reaction (MPCR) is a variant of PCR in which two or more amplicons can be amplified in the same reaction, therefore is a rapid and reliable way to study the level of cytokine gene expression. In this study, we established the MPCR assay for the study of porcine cytokine gene expression, in particular interferon-γ (IFN-γ), interleukin-10 (IL-10), IL-2 and IL-4, and used the established assay to study an *in vitro* effect of PRRSV on cytokine gene expressions in porcine PBMC.

Methods

Viruses. A wild-type US strain of PRRSV (SVI-275) and the CSFV, ALD strain, were kindly provided by Dr. S. Damrongwatanapokin at The National Institute of Animal Health, Bangkok, Thailand. The stock of PRRSV was prepared in MARC-145 cells and viral titers were determined as previously described (Thanawongnuwech *et al.*, 1998). The CSFV was propagated in a swine kidney cell line, SK-6 cells, and virus titers were determined as previously described (Suradhat *et al.*, 2001). The stock viruses were kept at –80 °C until needed.

Animals and immunization protocol. In the first experiment, blood samples were collected from three, five-week-old, non-vaccinated, crossbred pigs from a PRRSV-free commercial farm. In the second experiment, blood samples were collected from four crossbred pigs, obtained from the Faculty of Veterinary Science Research Farm, which had no serological evidence of exposure to PRRSV. Pigs were immunized with the lapinized Chinese-strain of CSF vaccine twice at 5 and 7 weeks of age, according to the routine vaccination program in the farm. Blood samples were collected when the pigs were approximately 16 weeks old.

Isolation and *in vitro* stimulation of porcine PBMC. Porcine peripheral blood mononuclear cells (PBMC) 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 PBMC were resuspended in RPMI 1640 (GIBCO/BRL, Rockville, MD) supplemented with 10% calf serum (Starrate, Australia), 2mM L-glutamine (GIBCO/BRL), 100 µM non-essential amino-acid (GIBCO/BRL), 1 mM sodium pyruvate (GIBCO/BRL), 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 µg/ml of amphotericin B (antibiotic/antimycotic solution; GIBCO/BRL). PBMCs, at a concentration of $6x10^6$ cells/ml/well of the 24-well plate, were cultured in the presence of antigen in a 5% CO_2, 37 °C incubator for 24-48 h as indicated in the text. The concentration of Concanavalin A (ConA, Sigma) used for *in vitro* culture was 10 µg/ml. The CSFV and PRRSV concentrations used for *in vitro* co-stimulation were 1 multiplicity of infection (m.o.i.), and 0.01 m.o.i., respectively. Following *in vitro* stimulation, cells were harvested and washed once with phosphate-buffered saline. Cell pellets were kept at –20 °C in the presence of RNAlater™ (Ambion, Austin, TX) until needed.

RNA extraction and reverse transcription. RNA was extracted from approximately $2x10^6$ cells using the Nucleospin® RNA II kit (Macherey-Nagel, Easton, PA), according to the manufacturer's instruction. Contaminating DNA was removed by DNase I treatment provided in the kit. At the final step, total RNA was eluted in 60 µl RNase-free water. Ten microliters of the total RNA from each sample was reverse-transcribed using Omniscript RT kit (Qiagen, Hilden, Germany) in a total volume of 20 µl reaction, according to the manufacturer's protocol, in the presence of 0.5 µg of random hexamers (Promega, Madison, WI) and 40 U of ribonuclease inhibitor (RNaseOUT™, Invitrogen, Carlsbad, CA). The RT reaction was carried at 37 °C for 60 min followed by the heat inactivation at 93 °C for 5 min and a rapid cooling on ice.

Multiplex PCR. All of the primers were designed specifically for MPCR to possess the same melting temperature (Tm) of 60 °C, based on the nearest neighbor analysis, in order to minimize the differences in amplification efficiency among the primers during MPCR. Sequences of the primer, the reference sequences, and expected sizes of the PCR products were given in Table 1. MPCR was performed in a total volume of 50 µl reaction containing 2 µl of the cDNA template, 10 µl of the primer mix (1 µl of each primer; see below), 10 mM dNTPs (Bio Basic Inc., Ontario, Canada), 2.5 U of *Taq* DNA polymerase (HotStarTaq DNA polymerase, Qiagen), 1.5X concentration of the PCR buffer provided with the enzyme. The amounts of each primer used in MPCR were empirically optimized according to the intensity of the band. The final concentration of the primers for GAPDH was 0.05 µM, 0.2 µM for IL-10, and 0.6 µM for IFN-γ, IL-2 and IL-4 primers.

The cycling parameters were consisted of 1) "hot start" at 95 °C for 15 min; 2) denaturing at 94 °C for 30 sec; 3) annealing at 55 °C for 45 sec; 4) extension at 72 °C for 45 sec; and 5) final extension at 72 °C for 5 min. The number of PCR cycle was optimized to assure that none of the products reached a plateau phase during the PCR amplification (data not shown). In this experiment, PCR amplification at 33 cycles was applied for further densitometric analysis. Following the MPCR, ten microliters of the PCR reaction was subjected to agarose gel electrophoresis using 2.5 % agarose (Sigma) in 1x TBE buffer (GIBCO/BRL)

in the presence of 0.5 μg/ml ethidium bromide (Research Organics, Cleveland, OH). A 100 bp DNA ladder molecular weight marker (GIBCO/BRL) was run in every gel as molecular weight standards.

Densitometric quantification of PCR product. Images of the MPCR products resolved in ethidium bromide-stained agarose gels were visualized under UV illuminator and digitally saved by the "Photo-print" photodocumentation system (Vilber Lourmat, France). The images were further processed for quantification of the band by densitometry using the Scion Image software (Scion corporation, USA). The expression level of each product was determined by normalizing its expression against the housekeeping gene, i.e. GAPDH, expression. The results were expressed as the percentage of the cytokine area/GAPDH area of the same sample and referred as % expression. When the PBMC populations were considered as a group, the values were averaged and the standard error of means (SEM) were calculated and expressed as Mean % expression ± SEM.

Verification of the PCR product by sequence analysis. Each cytokine and GAPDH PCR product was verified by sequencing. DNA fragments were cut from agarose gels and purified with Nucleospin® Extract kit (Macherey-Nagel) according to the manufacturer's protocol. The purified DNA was used as a template for the cycle sequencing reaction (DNA sequencing kit, Big Dye Terminator Cycle Sequencing, ABI PRISM PE Applied Biosystems, USA) and subsequently analyzed on an ABI prism 310 Genetic Analyser. The sequence identity was analyzed by using NCBI Blast software.

Results

Primer specificity and MPCR of cytokine mRNA transcripts

In the preliminary experiment, PBMC from naïve pig were cultured in the presence of 10 μg/ml ConA for 24 h and used as a positive sample for the establishment of MPCR. The ability of ConA to induce cytokine expression in pig had been already shown by the other group (Dozois *et al.*, 1997). The newly designed primer sets were able to amplified a single band of the expected size of porcine GAPDH, IFN-γ, IL-10, IL-2 and IL-4 from the positive sample (Fig. 1a). The specificity of the primer sets was confirmed by sequence analysis of each product (data not shown). Furthermore, the feasibility of MPCR was demonstrated when combining all the primer sets in a single-tube reaction (Fig 1b). Increased levels of cytokine gene expression were observed in ConA stimulated PBMC, whereas the level of GAPDH mRNA expression remained comparable in both unstimulated and ConA stimulated populations. It should be noted that in our system, unstimulated cells also produced considerable amount of cytokine transcripts after the culturing period, as shown in Fig. 1. In addition, these levels of background cytokine expression were somewhat varied among the pigs. Therefore, in order to eliminate this individual variability, the % expression of each cytokine from the unstimulated sample (i.e. background cytokine gene expression from the same animal) was subtracted from the % expression of the respective cytokine from the stimulated sample, prior to the calculation of the mean values. Therefore, the positive value represented an increase in % of the cytokine gene expression compared to the unstimulated sample from the same animal. The negative value indicated that the % expression was less than the % expression of the respective cytokine gene obtained from the unstimulated sample of the same animal.

An *in vitro* effect of PRRSV on porcine mRNA expression

Following the 24-hour- *in vitro* cultivation with PRRSV, an increase in IL-10 gene expression was the most apparent as compared to other cytokine genes. The IL-10 expression was significantly increased over the time of viral exposure (paired t-test, $p<0.05$). In this system, PRRSV seemed to have a minimal effect on IL-2 and IL-4 gene expressions in porcine PBMC and the effect of PRRSV on IL-10 and IFN-γ gene expressions appeared to be in a dose-dependent manner (Fig. 2).

The finding that PRRSV could enhanced the level of IL-10 expression in porcine PBMC raised a concern whether this observation was truly the effect of PRRSV or our newly established system was biased toward the detection of IL-10 gene. We, therefore, collected blood samples from pigs that were already primed with a classical swine fever vaccine (see methods). The isolated PBMC from each pig were further stimulated with the recall antigen, CSFV, in the presence or absence of PRRSV. In our previous work, the strong CSFV-specific IFN-γ production was detected from the PBMC of primed animals following the *in vitro* stimulation with CSFV using an ELISPOT assay (Suradhat & Damrongwatanapokin, 2000; Suradhat *et al.*, 2001). In addition, this experiment would allow us to explore if the presence of PRRSV had any effect on the recall antigen response.

Following the incubation period, the presence of PRRSV considerably upregulated the IL-10 gene expression (Fig. 3). This finding indicated that the effect of PRRSV on IL-10 gene expression was

reproducible even in pigs of different age and immune status. In fact, IL-10 gene seemed to be the most prominently expressed cytokine gene in the system. However, in the presence of recall antigen, CSFV, IFN-γ gene expression was predominantly enhanced, suggesting that the recall antigen response to CSFV was a Th1-like response and that our assay was not biased towards only the detection of IL-10 gene expression. Furthermore, when PBMC from primed animals were cultured in the presence of both PRRSV and CSFV, a significant decrease of IFN-γ gene expression (paired t-test, $p<0.05$) and a significant increase of IL-10 gene expression (paired t-test, $p<0.05$) were observed (Fig. 3a). These findings indicated that the presence of PRRSV in the culture significantly suppressed the recall response to CSFV. Similarly to the previous experiment, minimal effects of both viruses were observed on the levels of IL-2 and IL-4 gene expression (Fig. 3b).

Discussion

In this study we established the MPCR assay for detection of porcine cytokine gene expression. The RT-MPCR appeared to be a rapid and powerful tool to explore the dynamics of different cytokine productions from porcine samples. Furthermore, the use of densitometric analysis allowed an affordable semi-quantitative analysis of the level of mRNA transcripts, when target RNA levels were normalized with the reference level of the housekeeping gene. Previously, cyclophilin, β-actin or β_2-microglobulin genes have been used as references (Dozois *et al.*, 1997; Reddy *et al.*, 2000; Thanawongnuwech *et al.*, 2001). In the present report, we demonstrated that the GAPDH gene could be also used as a reference gene for study of cytokine gene expression in swine. Although increased cytokine gene expressions were observed in a ConA stimulated PBMC (Fig 1), it should be noted that the unstimulated cells also produced a considerable amount of the cytokine transcripts, in particular IL-10. This finding was not unusual, considering that several factors, both intrinsic and extrinsic, might influence the cytokine production in the culture system. The presence of IL-10 transcripts in unstimulated cells had also been previously observed (Dozois *et al.*, 1997; Thanawongnuwech *et al.*, 2001). In some cases, the level of IL-10 mRNA expression, analyzed by RT-PCR, was almost at the same level as with the ConA stimulated cells at all tested time points (Dozois *et al.*, 1997). Therefore, in our study, the "absolute increase" of the gene expression was determined by subtracting the background expression level of the cell control from the treatment expression level prior to analysis of the data. The increase in IL-10 gene expression was evident in PRRSV infected population, whereas IFN-γ gene expression were increased in the presence of a recall antigen, CSFV. In our study, the changes in IL-2 and IL-4 gene expression were minimal. An increase in IL-2 and IL-4 gene expression following ConA stimulation of porcine PBMC was previously reported using RT-PCR technique (Dozois *et al.*, 1997). The reason for this difference is not clear, however, the differences in the primer sensitivity and housekeeping gene used in the assay are likely to be the cause. Interestingly, our results was in the same line with the previous reported, in which a quantitative RT-PCR technique was used for studying of porcine cytokine gene expressions (Reddy *et al.*, 2000). In this study, several mitogens, including lipopolysaccharide, phytohaemagglutinin, hen egg white lysozyme and purified protein derivative of tuberculin, did not induce any detectable level of IL-2 and IL-4 gene expressions in porcine efferent lymph leukocytes. These results imply that pigs may not utilize the two cytokine as do humans and mice (Reddy *et al.*, 2000). Interestingly, in their experiment, the IL-10 gene expression was increased following 24 h of mitogen exposure and declined by 48 h, whereas in our study, the IL-10 expression continued to increase over the time in the presence of PRRSV (Fig. 2).

The observation that PRRSV upregulated the IL-10 expression *in vitro* is intriguing. There is at least one report, describing an increase in IL-10 mRNA expression from PBMC of piglets born from infected sow (Féng *et al.*, 2000). Although the RT-MPCR provides a rapid and convenient way of studying the cytokine gene expression, the relative amount of mRNA determined by RT-PCR does not necessarily reflect the relative amounts of the functional cytokine produced. Nevertheless, there are several *in vivo* findings suggesting that an increased IL-10 production *in vivo* may occur and contribute to the pathological outcome of PRRSV in infected animals.

IL-10 has been known to be the potent negative immunomodulator. The exploitation of IL-10 appears to be the common mechanism of immunosuppression by several intracellular pathogens specifically targeting macrophages for infection. Certain viruses induce IL-10 production, whereas others encode their own IL-10 to inhibit the host immune response and to hamper the viral clearance process (reviewed in Fickenscher *et al.*, 2002; Redpath *et al.*, 2001). Considering the restricted tissue tropism of PRRSV, it is possible that PRRSV also uses IL-10 for suppressing the host immune response. Induction of IL-10 production in an early stage of infection may enhance the viral survival within the host and delays the induction of protective immunity, which seems to be the case of PRRSV infection. IL-10 is known to

inhibit productions of proinflammatory cytokines including tumor necrosis factor-α (TNF-α), the major mediator of acute inflammatory response, and IL-12 from macrophages. The latter cytokine is known to play a key role in the development of cell-mediated immune responses (Abbas *et al*., 2000). The inhibitory effects of IL-10 on monocytes, macrophages, and dendritic cells have been extensively reviewed (Moore *et al*., 2001). A series of reports on kinetics of proinflammatory cytokine responses following PRRSV infection seem to support our view. Following PRRSV infection, production of TNF-α was almost undetectable (Van Reeth *et al*., 1999). Furthermore, the production of IFN-α was significantly suppressed in both PBMC and alveolar macrophages, and this inhibitory effect was not due to the cell death (Albina *et al*., 1998). The poor cytokine response agrees with the overall mild clinical course and minimal gross lung pathology (Van Reeth *et al*., 1999; Van Reeth & Nauwynck, 2000). Regardless of the virulence of infecting PRRSV, the effect of PRRSV on porcine macrophage bacteriocidal functions was previously demonstrated (Thanawongnuwech *et al*., 1998). Together, these findings support the notion that PRRSV may interfere with the overall function of the macrophage-like cells through the induction of immunosuppressive factor, such as IL-10, which may result in an alteration of a cascade of proinflammatory cytokine production. The inhibitory effect of IL-10 on the functions of antigen presenting cells (APC) may be one of the mechanisms for the delayed induction of both arms of protective immune responses to PRRSV, which is tended to develop long after the active phase of viral replication and viremia.

Our finding that PRRSV can also affect the cytokine production in the recall antigen response supports the immunosuppressive role of PRRSV. It is well established that IL-10 strongly inhibits the cytokine production and proliferation of $CD4^+$ cells, particularly the Th1 population via its downregulatory effect on APC function, resulting in inhibition of cell-mediated immune responses (Moore *et al*., 2001). The observed changes in IFN-γ and IL-10 gene expression may represent the direct effect of IL-10 on APC and T cell functions. Our finding has provided the evidence that the presence of PRRSV can significantly interfere with the production of IFN-γ in the recall response to CSFV (Fig. 3). Alteration of the magnitude and delayed T cell responses to pseudorabies virus vaccine in PRRSV-infected pigs has previously been observed (De Bruin *et al*., 2000). These findings support the role of PRRSV in interference of cell-mediated immune response in the host. Interestingly, infection or vaccination with PRRSV appear to decrease the efficacy of *M. hyopneumoniae* bacterin in *M. hyopneumoniae* challenged pigs (Thacker *et al*., 2000). Inhibition of the memory Th cell and/or effector cell functions by the viral-induced IL-10 may be one of the explanations for this decrease, since cell-mediated immunity is believed to play a significant role in respiratory defense against bacterial infections (Dunkley *et al*., 1995). The increase in IL-10 production may also explain an increased incidence of secondary infection in the lung following an episode of PRRSV infection apart from the direct effect of PRRSV on monocytes/macrophages.

In summary, our results imply that the enhanced IL-10 production may be one of the strategies used by PRRSV to regulate the host immune system. It would be interesting to explore if this phenomenon occurs *in vivo* following PRRSV infection and what is the mechanism of viral-induced IL-10 production. Knowledge in regard to the effect of PRRSV on the host immune system would be crucial for the development of the practical control strategy and in designing a safe and effective PRRS vaccine in the future.

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Legends

Figure 1 RT-PCR cytokine products from cultured PBMC. **Panel A** depicted the single PCR product of 1) GAPDH, 2) IFN-γ, 3) IL-10, 4) IL-2, 5) IL-4 from porcine PBMC cultured in the presence of ConA. **Panel B** depicted the PCR products from porcine PBMC cultured in the absence (-) or presence (+) of ConA for 24 h. A 100 bp DNA ladder molecular weight marker was included in the gel (M). The image shown is the representative image from 3 animals.

Figure 2 The means % expression of porcine cytokines in the presence of PRRSV. Porcine PBMC were cultured in the presence of 0.01 m.o.i. (white) or 0.001 m.o.i. (black) of PRRSV for 24 or 48 h prior to total RNA isolation and RT-MPCR.

Figure 3 The means % expression of (A) IFN-γ and IL-10 and (B) IL-4 and IL-2. Porcine PBMC were cultured with PRRSV, CSFV, or CSFV in the presence of PRRSV (CSFV+) for 24 hr prior to total RNA isolation and RT-MPCR.

Table 1. Oligonucleotide sequences designed for MPCR in this study

Gene specificity	Oligonucleotide sequences (5'-3')	GenBank Acc. No.	Product (bp)
GAPDH	F-TTCCACGGCACAGTCAA R-GCAGGTCAGGTCCACAA	AF017079	576
IFN-γ	F-CTCTCCGAAACAATGAGTTATACAA R-GCTCTCTGGCCTTGGAA	X53085	503
IL-10	F- AGCCAGCATTAAGTCTGAGAA R-CCTCTCTTGGAGCTTGCTAA	L20001	394
IL-2	F-TCTTGTGTTGCATTGCACTAA R-TCAGAGTTTTTGCTTTGACCTAA	X56750	280
IL-4	F- GGACACAAGTGCGACATCA R- GCACGTGTGGTGTCTGTA	X68330	186

F = forward. R=reverse

Figure 1

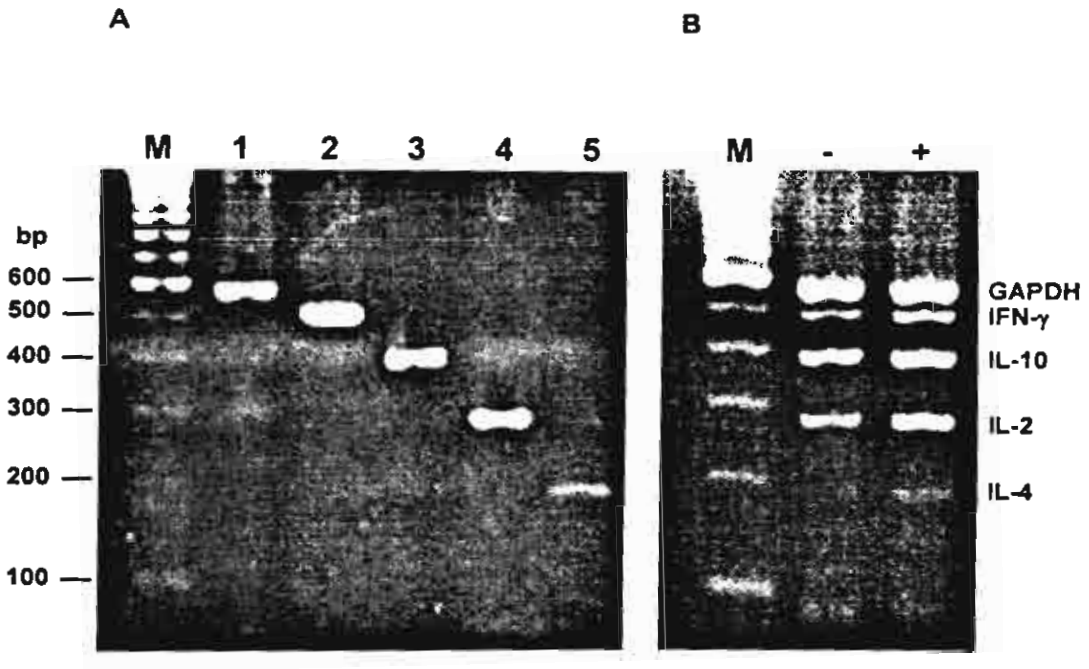


Figure 2

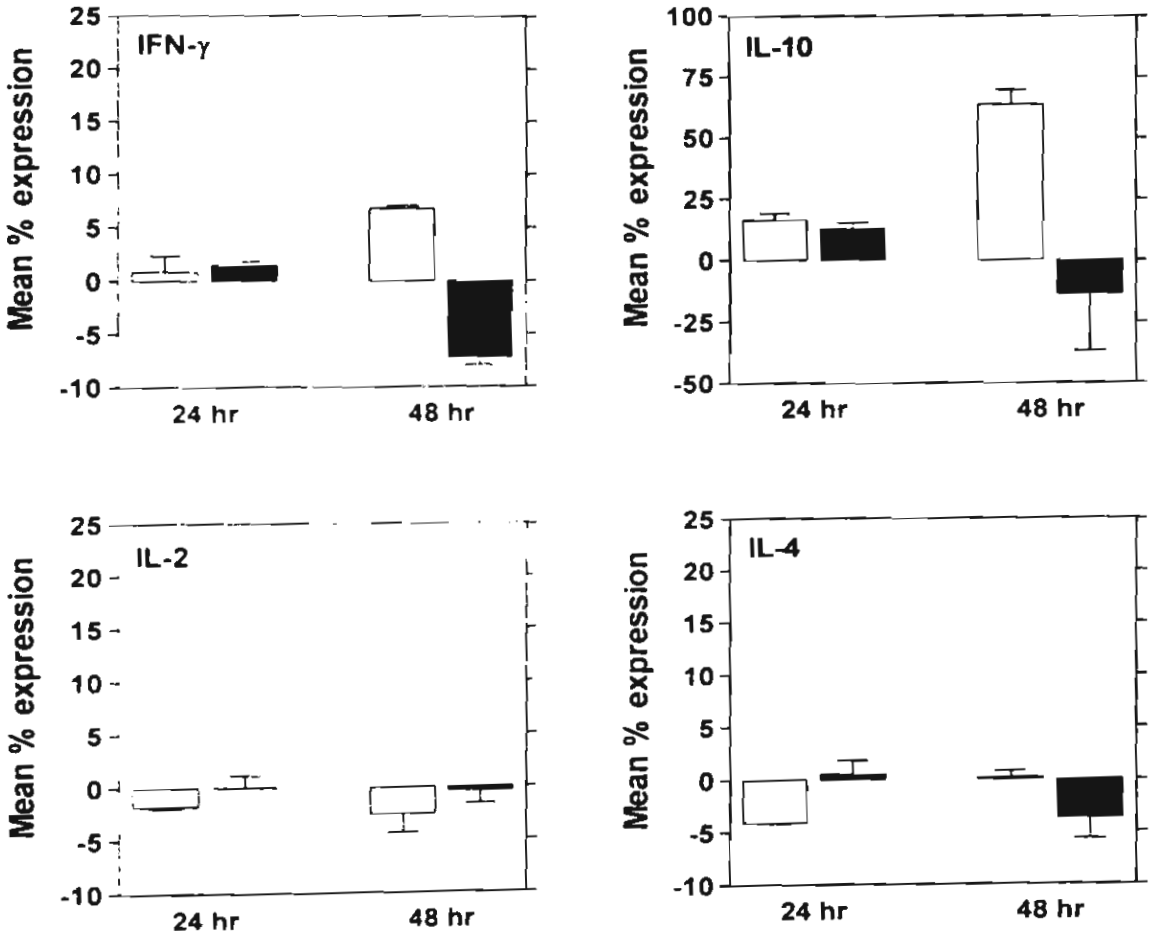
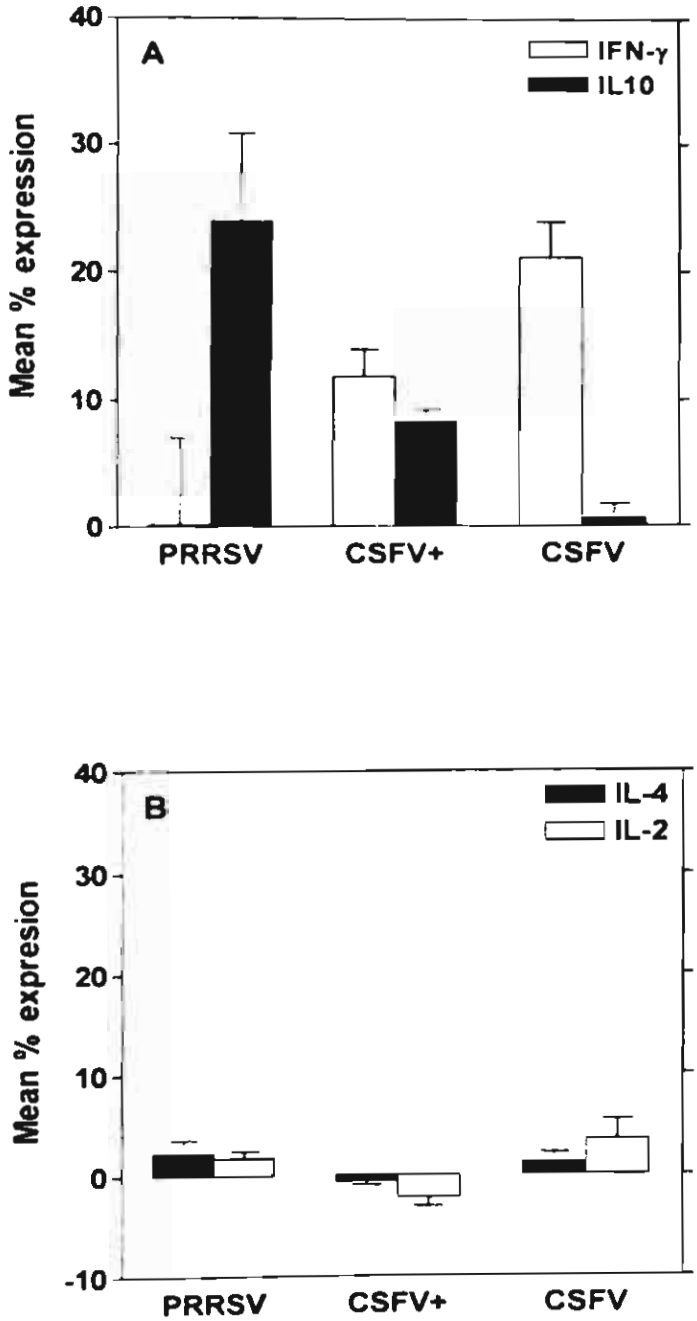

IFN-γ
Mean % expression
25
20
15
10
5
0
-5
-10
24 hr
48 hr
IL-10
Mean % expression
100
75
50
25
0
-25
-50
24 hr
48 hr
IL-2
Mean % expression
25
20
15
10
5
0
-5
-10
24 hr
48 hr
IL-4
Mean % expression
25
20
15
10
5
0
-5
-10
24 hr
48 hr

Figure 3


A
IFN-γ
IL10
Mean % expression
40
30
20
10
0
PRRSV
CSFV+
CSFV
B
IL-4
IL-2
Mean % expresion
40
30
20
10
0
-10
PRRSV
CSFV+
CSFV