รายงานฉบับสมบูรณ์

โครงการวิจัยเรื่อง
การใช้เซลล์เพาะเลี้ยงเยื่อบุมดลูกสุกรทดสอบความแตกต่างการตอบสนองต่อพีอาร์อาร์เอสไวรัสสายพันธุ์ชนิดรุนแรงต่างกัน

คณะผู้ดำเนินการวิจัย

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แหล่งทุนสนับสนุนงานวิจัย
งบประมาณแผ่นดินเงินอุดหนุนทั่วไปจากรัฐบาล ประจำปีงบประมาณ ๒๕๕๘
คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

กุมภาพันธ์ ๒๕๕๘

CONTENT

บทคัดย่อ

กลไกและพยาธิกำเนิดของการติดต่อโรคพีอาร์อาร์เอสจากแม่สู่ลูกยังไม่มีคำอธิบายที่แน่ชัดเนื่องจากยังขาดข้อมูลถึงความสามารถของการติดเชื้อที่ระบบสืบพันธุ์ เพื่อการศึกษาดังกล่าวด้วยการใช้เซลล์เยื่อบุมดลูกสุกรแบบเพาะเลี้ยงเบื้องต้นและทำการตรวจสอบการเปลี่ยนแปลงรูปร่างของเซลล์ (ซีพีอี) การตอบสนองของระบบภูมิคุ้มกันที่มีมาแต่กำเนิดโดยเฉพาะการต้านทาน และการหลั่งไซโตคายน์ชนิดอักเสบเมื่อได้รับเชื้อไวรัสพีอาร์อาร์เอสจากสายพันธุ์ยูเอส หรืออียูทางด้านท่อหรือด้านฐานเปรียบเทียบกัน การเปลี่ยนแปลงการปรากฏของโปรตีน และยีนของตัวรับชนิด CD151 CD163 และSn ต่อเชื้อไวรัสพีอาร์อาร์เอสซึ่งเป็นตัวการสำคัญที่ทำให้เซลล์สามารถติดเชื้อและมีความเสียหายเกิดขึ้นได้รับการตรวจสอบด้วยวิธี RT-PCR และอิมมูโนฮิสโตรเคมีตามลำดับ ผลการศึกษาพบว่าเซลล์เยื่อบุมดลูกสุกรปกติมีการปรากฏของตัวรับทั้งสองชนิดทั้งในระดับต่ำมาก การได้รับเชื้อไวรัสพีอาร์อาร์เอสทั้งสองสายพันธุ์เพิ่มการแสดงออกของตัวรับทั้งสองในระดับยีน และโปรตีน โดยที่สายพันธุ์ยูเอสจะเหนี่ยวนำให้แสดงออกได้มากกว่า($P<0.05$) เซลล์ที่ได้รับเชื้อมีการเปลี่ยนแปลงรูปร่างเป็นแบบพวงองุ่น และรวมตัวกันเป็นเซลล์ขนาดใหญ่หลังจากรับเชื้อ 2 วัน และพบความเสียหายของเซลล์หลังได้รับเชื้อ 6 วัน โดยที่เชื้อที่ให้ไปทางด้านทางด้านท่อทั้งสองสายพันธุ์สามารถติดต่อเข้าสู่เซลล์ของมดลูกได้หลังจากได้รับเชื้อ 2 วันโดยการตรวจสอบด้วยอิมมูโนฮิสโตรเคมี โดยที่สายพันธุ์ยูเอสเท่านั้นที่สามารถติดต่อจากทางด้านฐานและอยู่ในเซลล์ได้ถึง 6 วัน เชื้อไวรัสพีอาร์อาร์เอสทั้งสองสายพันธุ์จะกระตุ้นให้หลั่งไซโตคายน์ ชนิด IL-6, IL-8 or IFN-γ จากเซลล์เยื่อบุมดลูก โดยที่สายพันธุ์ยูเอสจะเหนี่ยวนำให้เซลล์ขับหลั่งได้มากกว่า ($P<0.05$) อย่างไรก็ตามถ้าเซลล์ได้รับการติดเชื้อทั้งสองสายพันธุ์ทางด้านฐานพบว่าทำให้เกิดการยับยั้งการหลั่ง IL-8 ออกมาทางด้านฐานของเซลล์ ในด้านการศึกษาความต้านทานของเยื่อบุโดยการวัดความต้านทานทางไฟฟ้าของเซลล์เยื่อบุ (TER) พบว่าค่า TER ลดลงเมื่อเซลล์ได้รับเชื้อไวรัสสายพันธุ์อียูไปเพียง 2 วัน แต่จะกลับมีค่าเท่ากับก่อนได้รับการติดเชื้อหลังจากได้รับเชื้อไปแล้ว 6 วัน เป็นที่น่าสนใจว่าเซลล์ที่ได้รับการติดเชื้อไวรัสสายพันธุ์อียูตั้งแต่ 4 วันมีค่า TER ที่สูงมากกว่าเซลล์ปกติ หรือเซลล์ที่ได้รับเชื้อสายพันธุ์ยูเอส ($P<0.05$) การศึกษาครั้งนี้บ่งชี้ให้เห็นว่าเซลล์เยื่อบุมดลูกที่มีการแสดงออกของตัวรับเชื้อไวรัสไวรัสพีอาร์อาร์เอสในปริมาณน้อยสามารถมีการติดเชื้อได้ และการติดเชื้อเชื้อไวรัสสายพันธุ์ยูเอสสามารถกระตุ้นให้มีการตอบสนองของระบบภูมิคุ้มกันได้ดีกว่าไวรัสสายพันธุ์อียู โดยที่ความสามารถในการเปลี่ยนแปลงการแสดงออกของตัวรับเชื้อไวรัสพีอาร์อาร์เอสที่เกิดขึ้นภายหลังจากการได้รับการติดเชื้อนั้นอาจเป็นเป้าหมายที่สำคัญต่อการรักษา หรือการที่จะนำเซลล์ของเยื่อบุมดลูกมาเป็นเซลล์ที่ใช้เพาะเลี้ยงไวรัสพีอาร์อาร์เอสเพื่อการผลิตวัคซีนต่อไป

ABSTRACT

Pathogenesis of Porcine Reproductive and Respiratory Syndrome (PRRS) horizontal transmission have not been explained. One reason is lack of information about the PRRS virus (PRRSV) susceptibility in the reproductive system. Using the primary porcine glandular endometrial (PE) cells, the cytopathic effects (CPE) after infected with PRRSV was investigated for the susceptibility of PE. Innate immunity response (i.e. epithelial resistance and pro-inflammatory cytokine secretion) to PRRSV infections with different strain US/EU and route basolateral/apical were compared. PRRSV and its specific receptor mRNA and protein CD151, CD163 and/or Sn expression were presently examined by immunohistochemistry (IHC) and qRT-PCR, respectively. The data demonstrated that normal PE cells showed low level of CD163 and Sn mRNA and protein expression. Infection with EU and US strains up-regulated CD163/151 and Sn mRNA and protein expressions in PE cells ($P<0.05$). US strain infection induced expressed PRRSV protein on PE cells higher than the EU strain ($P<0.05$). The syncytial-like or grape-like particles CPE were obviously seen since the 2 dpi followed by the cell lost at the 4-6 dpi in PE cells infected with EU or US strains. PRRSV replicated in PE detected by IHC were revealed in PE infected with EU or US strains at apical but not basolateral side starting at 2 dpi. However, US strains PRRSV at basolateral can infect and replicate in PE cells until 6 dpi. All PRRSV modified the secretion of IL-6, IL-8 or IFN-γ with the difference degree. US strain has higher stimulating effects on cytokine secretion than EU strain in PE cells (*$P<0.05$*). However, the basolateral infected with EU or US eliminated IL-8 secreted to the basolateral side of PE. Epithelial barrier determined by TER was decreased early only at 2 dpi in EU strain infection. However, the decreased TER affected by PRRSV infection returned to normal with 6 days. In addition, TER of PE cells infected by EU strain had significantly higher than normal or US-infected cells. In summary, PE cell, which express low level of specific receptors are able to be infected with PRRSV. However, infection with US strain the virulent strain revealed higher innate immunity response than EU strain in PE cells. Modification of PRRSV mediator expression in PE cells responded to PRRSV may become the targets for anti-viral agents and useful for viral isolation during vaccine developing processes.

INTRODUCTION

Porcine reproductive and respiratory syndrome virus (PRRSV)-induced reproductive problems are characterized by embryonic death, late-term abortions, early farrowing and increase in the number of dead and weak fetuses. Recent findings indicate that the endometrium and placenta are involved in the PRRSV passage from mother to fetus and that virus replication in the endometrial/placental tissues can be the actual reason for fetal death. Better understanding of these phenomena, i.e. the specific route of viral transmission (mucosa vs. basolateral blood-borne route), specific PRRSV receptor and mucosal immunological response, may facilitate preventive strategies.

The presence of PRRSV target cells in the endometrium and placenta may be essential for virus passages from mother to fetus. In line with this, the highest number of $CD163^+$ and Sn^+ cells (target cells for PRRSV infection) is observed in the endometrium and placenta collected at 90-110 days of gestation than at earlier stages. The abundance of cells that are highly susceptible to the virus in the placenta during late gestation may in part explain why congenital PRRSV infection is mostly restricted to the end of gestation. A previous challenge experiment revealed that the endometrial environment may also play an important role in the establishment of placental and transplacental PRRSV infections. The still unknown factors that prevent or block PRRSV replication in the endometrium and the not sufficient number of susceptible cells in the placenta might join forces and cause resistance to placental/transplacental PRRSV infection before 90 days of gestation. Therefore, it is of interest to firstly investigating whether or not porcine endometrial cell may be the target or reservoirs of PRRS viral infection leading to PRRS replication by examining the expression of CD163 and Sn correlation with the numbers of PRRS viruses in the cells.

Cytokines and chemokines play a key role in the regulation of the innate, humoral (T-helper 2 [Th2]) and cellular (T-helper 1 [Th1]) immune responses [1]. Early cytokines, including the type I interferons and pro-inflammatory cytokines (interleukins 1 (IL1), IL6 and tumor necrosis factor-alpha (TNFα), and late cytokines such as interferon-gamma (IFN-γ), are important regulators of adaptive immune responses [2]. An important chemokines are interleukin 8 (IL8 or CXCL8), a potent recruiting of neutrophils to sites of infection, and chemokine ligand 2 (CCL2), which induces the migration of monocytes from blood to become tissue macrophages [3]. The recent study indicated that endometrial cell culture without immune cells have ability to secrete all that types of cytokines to protect them from invading microorganisms.

Taken together, the present study aims to investigate the protective response of primary porcine endometrial cell culture (PE cells) by examining the expression of PRRS viral receptors and secretion of cytokines in the presence or absence of PRRS viral infection. The comparison between the route of infection (apical vs. basolateral), non-virulent vs. virulent strain, EU and US strain is aimed to be determined.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM), Dulbecco's phosphate buffer saline (DPBS), phenol red-free DMEM, fetal bovine serum (FBS), 0.05% trypsin-0.53 mM ethylenediaminetetraacetic acid (EDTA), kanamycin, penicillin-streptomycin and fungizone were purchased from GIBCO BRL (Grand Island, NY). Charcoal-stripped FBS was purchased from Biowest Co., (Miami, FL).

Cell isolation and culture

Porcine endometrial tissues of slaughtered finishing gilt (Thai commercial breed) were obtained from governmental qualifying slaughter house in Bangkok, Thailand. During transportation, tissues were maintained in the ice-cold porcine's ringer solution containing 130 NaCl, 6 KCl, 3 $CaCl_2$, 0·7 $MgCl_2$, 20 $NaHCO_3$, 0·3 NaH_2PO_4, 1·3 Na_2HPO_4, pH 7.4. Preparation protocol of PE cell was followed Deachapunya and O'Grady (10) method. Briefly, the tissue was cut into small pieces in Ca^{2+} and Mg^{2+}-free PBS and digested 24 hours at 37°C with 0.2% collegenase type I (Gibco, Invitrogen, CA, USA). Digested tissue was filtered through a mesh to remove stromal cells. The filtrate was incubated at room temperature for 15 minutes to collect the pellet for 3 times. According to this step, the PE cell masses were isolated by gravitational sedimentation. Following the sedimentation, the cell pellets were resuspended with growth media, 10% fetal bovine serum (FBS) in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Invitrogen, CA, USA) containing 100 U/ml penicillin, 100 µg/ml, streptomycin, 100 µg/ml kanamycin, 1% non-essential amino acids and 10µg/ml insulin, and plated in 100 mm^2 dish. After incubation in 5% CO_2 at 37°C for 48 hours, PE cells were pipetted to remove the excessive glands and refreshed media. Partial trypsinization with 0.25% trypsin/EDTA was performed next 48 hours to remove the remaining stromal cells. PE cells were subcultured and plated at 90% confluent prior to perform the experiment.

Isolation of PRRSV

Thai PRRSV field isolated, EU and US strains, were obtained from field infected lung of piglets. Infected lung tissues were cut into 2 g and homogenized with 15 ml of cold DMEM using glass Homogenizer. Tissue homogenate was centrifuged 10,000g at 4°C for 10 mins to remove cell debris and collect the supernatant. This step was performed continual 2 times. Then the supernatant was filtered with 0.2 μm filter (Corning, NY, USA) and diluted 1:1 with DMEM prior to PE cell infection.

Infection PE cell with PRRSV

1x 10^6 of PE cells were seeded in 24 mm^2 microporous membranes and 25 cm^2 flasks (Costar®, Corning, MA, USA). PE cells were cultivated with 5% FBS in DMEM (maintaining medium). After incubation for 48 hours, PE cells were replaced with fresh media. Media were refreshed every 48 hours for 7 days. At day 7, PE cell was infected apically/basolaterally on the membrane, and PE cells in the flask were infected with mock infection, PRRSV EU or US strain (n=3).

PE cells were removed media and washed with PBS before infection with PRRS. Subsequently, apical infection performed by adding 1 ml DMEM containing PRRS EU or US strain at the apical side of the membranes. Basolateral infection performed by adding 2 ml DMEM containing PRRS EU or US strain at the basolateral side of the membranes. Mock infection and non-infection side were performed by adding DMEM 1 ml at the apical or 2 ml at the basolateral side of the membranes. PRRSV infection in 25 cm^2 flask was performed by adding 5 ml of virus in DMEM as above. Then infected PE cells were incubated in 5% CO_2 at 37℃ to permit the complete viral infection. After 1 hour incubation, PE cells were washed with DMEM and maintained by maintaining medium for 2-6 days. Media were collected from apical and basolateral sides at 0, 2, 4, 6 day post infection (dpi) then replaced with fresh maintaining media every 48 hours. The microporous membranes were also collected and fixed in 4% paraformaldehyde at 2, 4, 6 dpi prior to perform immunohistochemistry.

RNA isolation

At 4 day post infection, total RNA was extracted from infected PE cells cultivating in 25 mm^2 flasks using TRIzol® reagent (Invitrogen™, CA, USA) according the manufacturer's instruction. PE cells in the flask were trypsinized with 0.25% trypsin/EDTA and centrifuged to collect PE cell. Briefly, 200 μl of TRIzol® reagent was added to each sample. Then the

sample was added chloroform 40 µl and centrifuged to separate RNA. RNA was collected from the transparent layer of the sample and added 100 µl of isopropanol to precipitate RNA pellet. The RNA pellet was washed 2 times with 200 µl of 70% ethanol. The final total RNA pellets were air dried for 1 hour and eluted in 20 µl nuclease-free water (Bio-rad, Inc., CA, USA). Total RNA concentration was measured at an optical density (OD) 260 nm using NanoDrop equipment (NanoDrop 2000c, Thermo scientific, Thermo Fisher Scientific, MA, USA), and purity was determined by calculation of the OD_{260}/OD_{280} ration.

cDNA synthesis

Reverse transcription was performed using a cDNA synthesis kit (iScript^{TM,} Bio-rad, Inc., CA, USA) in a 20 µl reaction mixture containing 3 µg of total RNA, 2 µl Oligo dT primer, 4 µl 5x iScript reaction mix, 1 µl iScript reverse transcriptase and nuclease-free water. The cDNA synthesis was accomplished in thermocycler (Biometra, Göttingen, Germany) according the manufacturer's protocol. The concentration of cDNA was determined at OD 260 nm using NanoDrop equipment (NanoDrop 2000c, Thermo scientific, Thermo Fisher Scientific, MA, USA).

Determination of PRRSV mediator gene expression using qPCR

Real-time PCR was performed using a GeneOn real-time PCR kit (GeneOn, Deutschland, Germany) in a final volume 20 µl consisting of 3 µg of cDNA template, one set of primers (1µl each, final concentration of 0.5 µM), 10 µl of E4 and DW added up to 20 µl. The reaction was performed in theremocycler (CFX384 Touch^{TM}, Bio-rad, Inc., CA, USA). All PCR procedures consisted of initial denaturation at 95℃ for 3 mins, followed by 40 cycles of amplification steps, including denaturation at 95℃ 20 Sec, annealing at 60℃ 30 Sec and extension at 72℃ 30 Sec respectively. The amplification products were confirmed the specificity by performing 1.5% agarose gel (Seakem, ME, USA) electrophoresis and melting curve analysis. The amount of PRRS mediator mRNA expressions was normalized to GAPDH mRNA expression as an endogenous control, and data were shown as fold changes using $2^{-\Delta\Delta Ct}$. All primer sets were designed by iSciencetech (iScience technology, BKK, Thailand). The sequences of primer sets were shown in Table 1.

Table 1. Sequences of porcine specific real-time PCR primers

Gene	Primer sequences (5' —> 3')	Accession number	Product size (bp)
CD151	F: TGTGTGCAGGTGTTCGGCAT R: TCAGCGCATCCTGAGAAGCT	NM_001243865.1	125
CD163	F: AATTCCAGTGTGAGGGGCAC R: AGCGGATTTGTGTGTATCTTGAG	HM991330.1	123
GADPH	F: GGACCAGGTTGTGTCCTGTGA R: TCCACCACCCTGTTGCTGTAG	NM_001206359.1	143

Determination of PRRSV and PRRSV mediator protein expression using immunohistochemistry

Related PRRSV and PRRSV entry mediators, including CD163 and sialoadhesin were observed the expression via immunohistochemistry (IHC) at 2, 4, 6 dpi. The collected membranes were washed with PBS prior to blocking endogenous peroxidase with 10% H_2O_2 in methanol and blocking non-specific with 4% goat serum in PBS. All of samples were performed duplicately by adding primary antibodies adding and/or PBS containing 1% BSA and 0,1% tween (negative control). The membranes were added primary antibody, including goat-anti-CD163 (Santa Cruz biotechnology, Santa Cruz, CA, USA) dilution 1:25, mouse-anti-sialoadhesin (Serotec®, Bio-rad, Inc., CA, USA) dilution 1:25, and incubated in 4℃ overnight. To detect PRRSV expression, the membranes were incubated with rabbit-anti-PRRSV dilution 1:100 (Biorbyt, UK) at 4℃ for 4 hours. Then membranes were triplicately washed with 0.1% tween in PBS and incubated with HRP-streptavidin secondary antibodies, using mouse-anti-mouse/rabbit IgG (Vectastain, Vector laboratories, Inc., CA, USA) dilution 1:2000 or donkey-anti-goat (Santa Cruz biotechnology, Santa Cruz, CA, USA) 1:200, for 1 hour at room temperature. Membranes were triplicately washed with PBS, then added conjugated HRP and incubated 30 mins at room temperature. Finally, they were stained with DAB substrate and counter stained with hematoxillin (Histostain-SP, Invitrogen, CA, USA). The processed sample was mounted on the slide and sealed cover slip with mounting solution (Histostain®, Invotrogen, USA). PRRSV and PRRSV mediator protein expressions of the sample were observed under light microscope. The positive result was subtracted background from the negative control. Golden brown color expressions in PE cells were counted as

positive cells. Expressions of PRRSV and PRRSV mediators were calculated and reported as % immunoreactivities per field (n=3).

Measurement of transepithelial electrical resistance (TER)

Transepithelial electrical resistance (TER) was performed to determine the permeability of tight junction lining at the uppermost portion of endometrial epithelial cells. Following the subculture of PE cells on the transwell inserts, the TER was periodically measured by using EVOM^{2TM} electrode connected to volt-ohmmeter (World Precision Instruments, Inc. Sarasota, FL) over 24 h intervals before and after drug treatment. To monitor the changes in TER by PRRSV stimulation, the inserts containing the PE cells grown in culture medium for 2 days were measured for TER. The TER was measured before (0 min) and at 30 min, 1, 2, 24 and 48 h after infection with PRRSV. Percent changes of TER at each time point from the starting point were calculated and analyzed.

Measurement of cytokines

PE cells of 10^6 cells were seeded on 6-well plate containing 5% FBS for 96 h. Then phenol-red free charcoal stripped-test medium containing drug or vehicle treatment was substituted and cultured for 48 h. During this process, 500 µl of cell-culture test medium from the apical site was collected at day 6 of infection for detecting of interleukin-1β (IL-1β), interleukin-8 (IL-8), interleukin-6 (IL-6), interleukin-10 (IL-10), tumor-necrosis factor- (TNF-α) and interferon gamma (IFN-γ) production by Enzyme-linked immunoabsorbent assay (ELISA) which was conducted by ELISA kit DuoSet ‹(R&D system Minneapolis, MN, USA). Following the manufacturer's protocol, after coating ELISA plate with capture antibody overnight into 96 well-ELISA plate at 4^{o}C, three-time washing with washing buffer was pursued. Then the reagent diluent containing with 1% bovine serum albumin (BSA) was applied for blocking non-specific binding, and three-time washing step was repeated. 50 µl of sample media and standard of studied cytokines were coated and incubated for 2 h at room temperature. The biotinylated detection specific antibody conjugated with streptavidin-horseradish peroxidase (HRP) was then applied to each well. Substrate solution and TMB were fallowing applied and incubated for 30 min prior to H_2SO_4 was added to stop the reaction. The concentration of studied cytokine was determined by measuring optical density (O.D.) at 450 nm and 620 nm. The minimal level of detection of all cytokines was 100 ng/ml,

25 ng/ml and 2 μg/ml, respectively. All measurements of cytokine assay were duplicates. In addition, the experiments were repeated at least three times.

Data analyses

All data were demonstrated as mean±SEM. Statistically analyzed using one way ANOVA to compare differences between strains in particular infection date. $P<0.05$ was considered as significant difference between means. If data were not passed normality test, the Newman-Keul method was used.

RESULTS

Susceptibility of PE cells to PRRSV

To determine the susceptibility of PE cells to PRRSV infection, PE cells were observed histologically changes every 48 hours. As figure 1 shown, the mock-infected PE cells were not observed the changes during 0-6 days post infection (dpi). The cytopathic effects (CPE) were obviously seen since 2 dpi in PE cells apically and basolaterally infected with EU or US strains. Accumulation of PE cells, which may be due to epithelial hyperplasia was demonstrated in all PE cells infected PRRS at 2 dpi. However, cell loss with CPE which is structural changes of PE cell resulting from viral infection was intensely observed in 4-6 dpi infected basolaterally with all PRRSV (Fig.1L). In addition, grape-like particles with CPE and cell loss were demonstrated in 6 dpi at the PE infected with PRRSV at the apical side (Fig.1P). On the last day of infection (6 dpi), the PE cells infected with EU strain were dead and detached from the membranes.

To confirm the PRRSV infection, PE cells, mock-infected, EU strain infected or US strain infected, were performed immunohistochemistry to observed PRRSV expression at 2, 4, 6 days post infection (dpi). There were no PRRSV expressions in mock-infected PE cells during 0-6 dpi. The PE cells apically infected with EU or US strain were an observed PRRSV expression at 2, 4, 6 dpi (Fig. 2). At 2 to 4 dpi, apical infection with US expressed PRRSV in PE cells higher than the EU strain infection ($P<0.05$). For basolateral infection, only PE cells infected with a US strain expressed PRRSV. PRRSV expressions were not observed in a PE cell at any date of PRRSV EU strain basolateral infection. At 6 dpi, the expression of PRRSV in PE cells were not significant differences between the apical infection with EU or US strain. PRRSV was not expressed in PE cells any group of basolateral infection at 6 dpi.

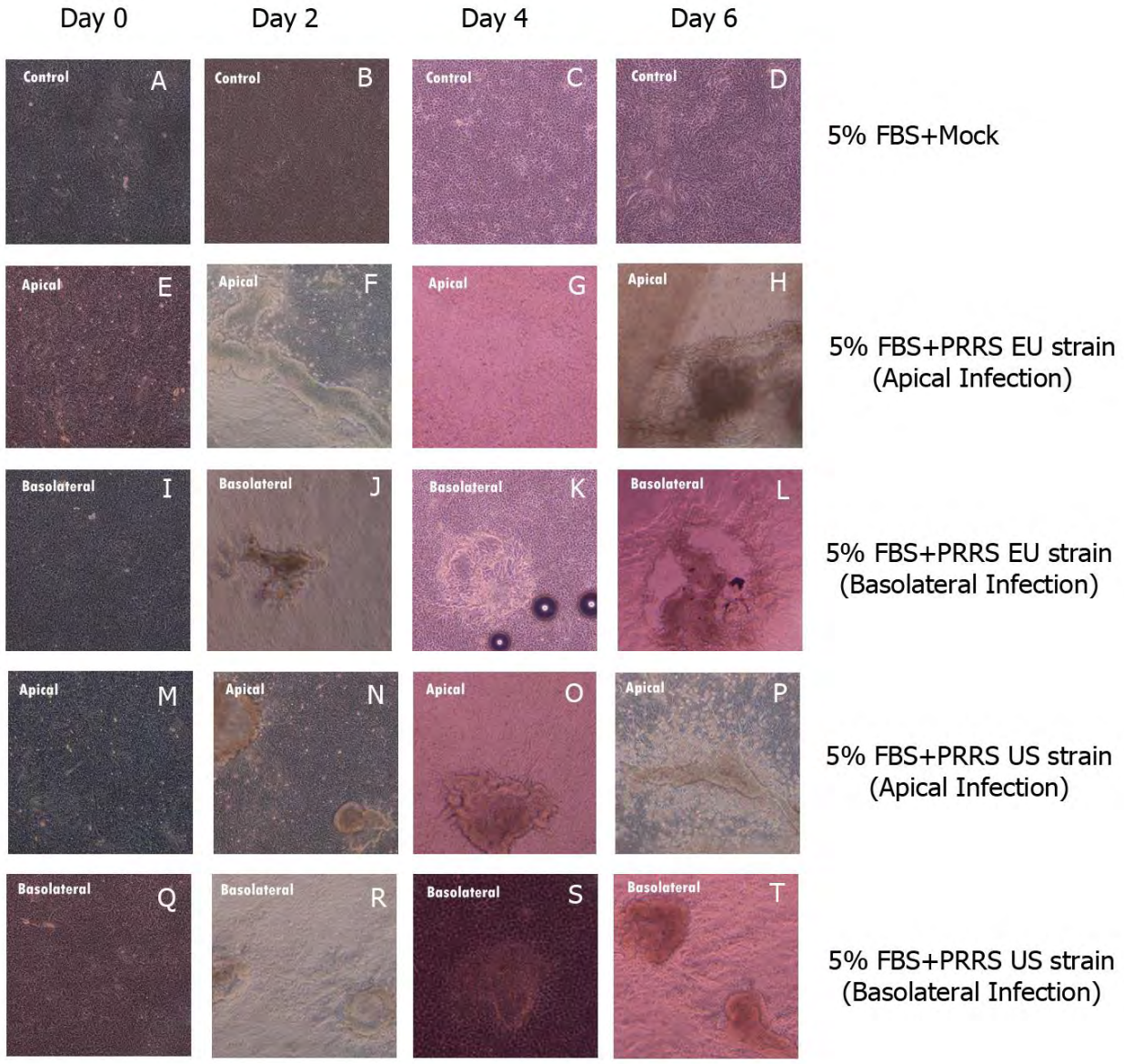

Figure 1 Histological changes of porcine endometrial cell culture (PE) in the control **(A-D)** compared with PE cells infected with EU strained PRRS at the apical side (E-H) and basolateral side (I-L) or US strained PRRS at the apical side (M-P) and basolateral side (Q-T). Photograph of observed cells was taken at 0, 2, 4 and 6 days after infection.

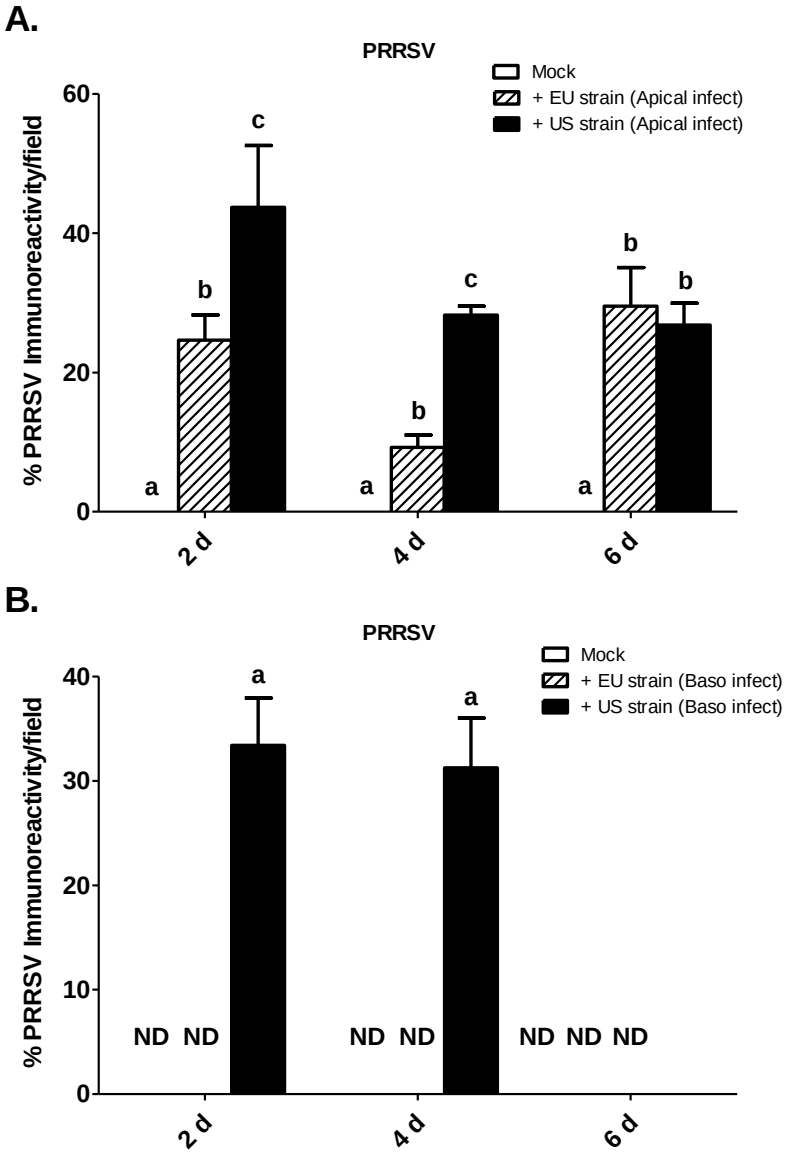

Figure 2 Assessment of PRRSV immunofluorescence in porcine endometrial cell culture (PE) in the control compared with PE cells after infection with EU or US strained PRRSV at the (A) apical side or (B) basolateral. Data was represented in means±S.E.M of a percent of PRRSV immunoreactivity/field. Experiments were conducted in triplicate. Bars with different letters are significantly different at the value of $p<0.05$ analyzed by one-way ANOVA and Newmans-Keul post-hoc comparison at each site of infection. ND is noted when the PRRSV immunoreactivity could not be detected.

Effects of PRRSV on cytokine secretion by PE cells

Interleukin-6 (IL-6) secretion

After seeding PE cell in insert filter and reaching confluence, the cultured medium (5%FBS) in apical site was collected at 0, 2, 4 or 6d for detecting IL-6 secretion by ELISA, the preliminary result showed that in normal condition (5%FBS) IL-6 secretion by PE-cell was significantly increased in a time dependent for both sides of PE cells. Therefore, the comparison of IL-6 secretion in the absence and presence with PRRS infection was analyzed. Apically infection with US strained PRRS significantly stimulated the IL-6 secretion and accumulation on the apical higher than any treatment (Fig.3). Even though, IL-6 secretion accumulated on the basolateral sides after apically infection with US strained PRRS seems to be increased, the statistically increased could not reach the successfully significant from the control (Fig.3A).

However, all basolaterally PRRS infection, which simulates the circulatory-route viral infection depressed almost the IL-6 secretion of PE cells (Fig. 3B). Interestingly, the impact of basolaterally PRRS infection seems to eliminate the secretory function of PE cells.

Interleukin-8 (IL-8) secretion

Similar to IL-6 secreted by PE cells, IL-8 secretion by PE-cell was also time dependent. Infection with US strained PRRS either apical or basolateral sides starting at 2 days of infection significantly stimulated the IL-8 secretion higher than control or EU strained PRRS (Fig.4). Even though, the increased IL-8 only at the apical sides was observed in response to PRRS infection (Fig. 4).

In the present study, it is interesting that the increased IL-8 secretion stimulated by PRRS seems to be very early, since there were no significantly differences of IL-8 secretion between absence or presence of PRRS after 6 days of infection.

IFN-γ secretion

In the PE cells, IFN-γ secretion seems to be very low. However, time-dependent secretion of IFN- γ was also observed similar to IL-6 or IL-8 secretion. In contrast to IL-6 and IL-8 secretion, the increase of IFN-γ accumulated on the apical side of PE response PRRS infection was observed (Fig.5). It has to be noted that only the apically infection with EU strained PRRS or basolaterally infection with US strained PRRS was able to increase IFN-γ secretion in the present study. However, the inhibition of IFN- γ secretion affected by PRRS

infection was demonstrated when PE cells infected with EU strain at the circulatory basolaterally route (Fig.5B).

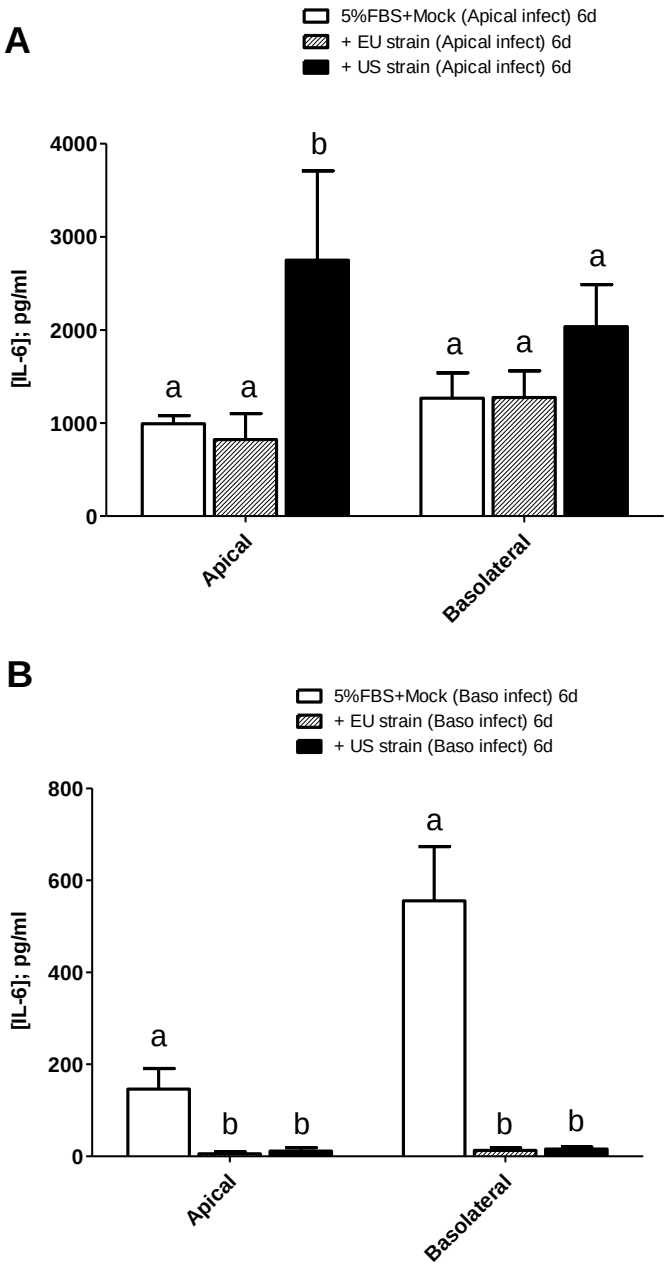

Figure 3 The secretion of IL-6 from PE cells in response to PRRS viral infection at **(A)** the apical site or **(B)** the basolateral site. Cultured medium bathed at the apical and the basolateral site collected from the inserted filter before and after 6 days (6d) of PRRSV inoculation was assayed for IL-6 secretion by ELISA. Data was represented in means±S.E.M of concentration of IL-8 in pg/ml. Experiments were conducted in triplicate. Bars with different letters are significantly different at the value of $p<0.05$ analyzed by one-way ANOVA and Newmans-Keul post-hoc comparison at each site of infection.

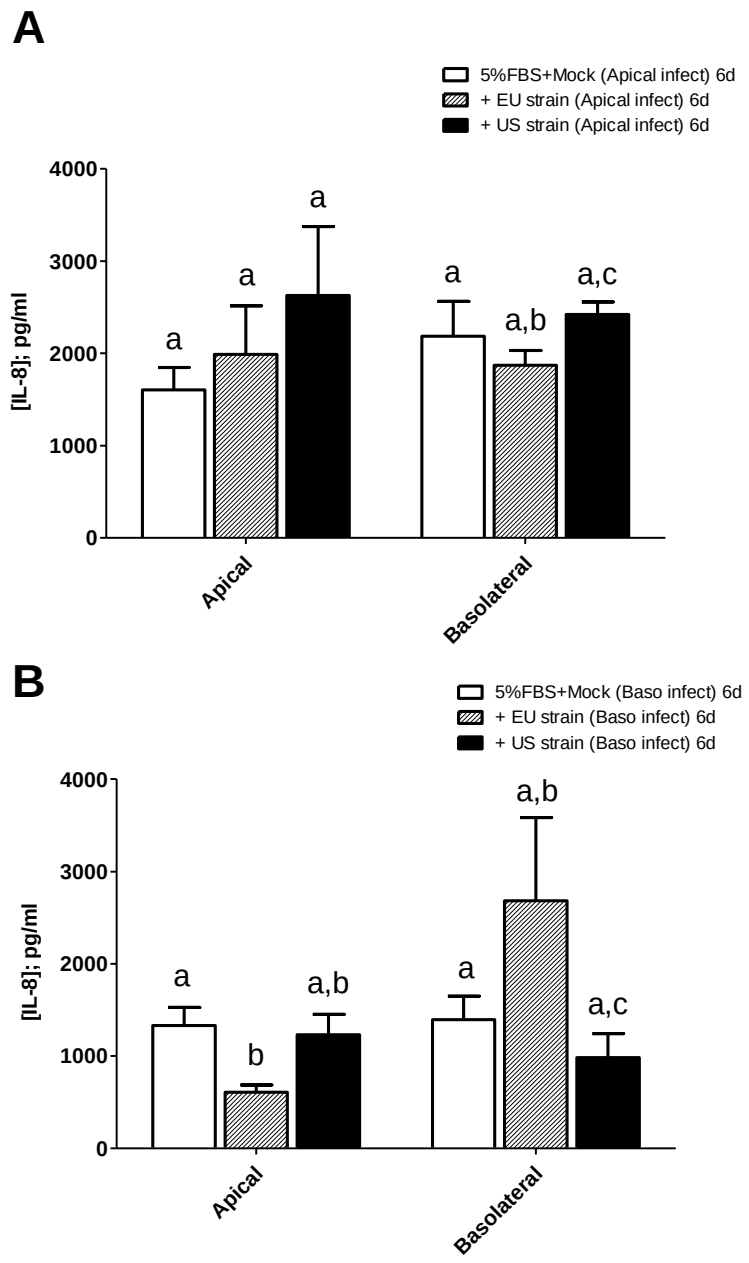

Figure 4 The secretion of IL-8 from PE cells in response to PRRS viral infection at **(A)** the apical site or **(B)** the basolateral site. Cultured medium bathed at the apical and the basolateral site collected from the inserted filter before and after 6 days (6d) of PRRSV inoculation was assayed for IL-8 secretion by ELISA. Data was represented in means±S.E.M of concentration of IL-8 in pg/ml. Experiments were conducted in triplicate. Bars with different letters are significantly different at the value of $p<0.05$ analyzed by one-way ANOVA and Newmans-Keul post-hoc comparison at each site of infection.

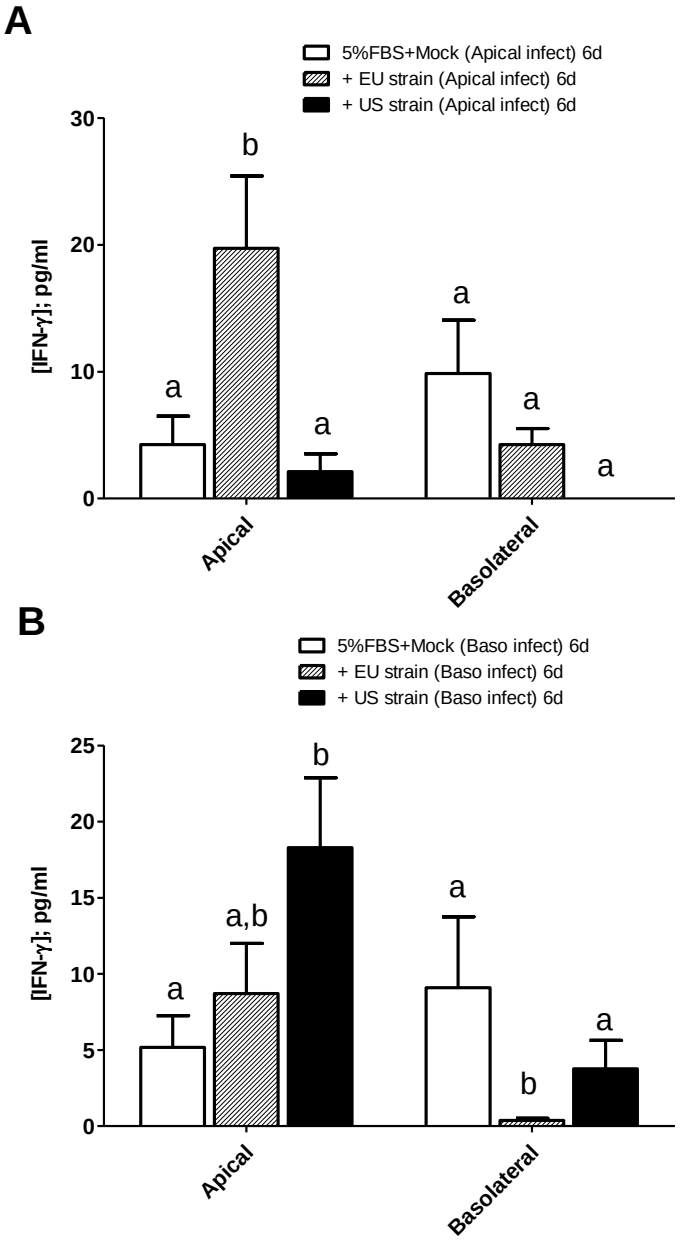

Figure 5 The secretion of IFN-γ from PE cells in response to PRRS viral infection at (A) the apical site or (B) the basolateral site. Cultured medium bathed at the apical site and basolateral collected from the inserted filter before and after 6 days (6d) of PRRSV inoculation was assayed for IFN- γ secretion by ELISA. Data was represented in means±S.E.M of concentration of IFN-γ in pg/ml. Experiments were conducted in triplicate. Bars with different letters are significantly different at the value of $p<0.05$ analyzed by one-way ANOVA and Newmans-Keul post-hoc comparison at each site of infection.

Electrophysiological changes of porcine endometrial cells response to PRRS viral infection EU strain vs US strain

In the PE cells, the epithelial tissue resistance of PE cells infected with PRRS seems to be differences from the control PE. Briefly, PE cells infected with a US strain were lower than those of controls, whereas PE cells infected with the EU strain were significantly increased to be higher than any other groups (Fig.6)

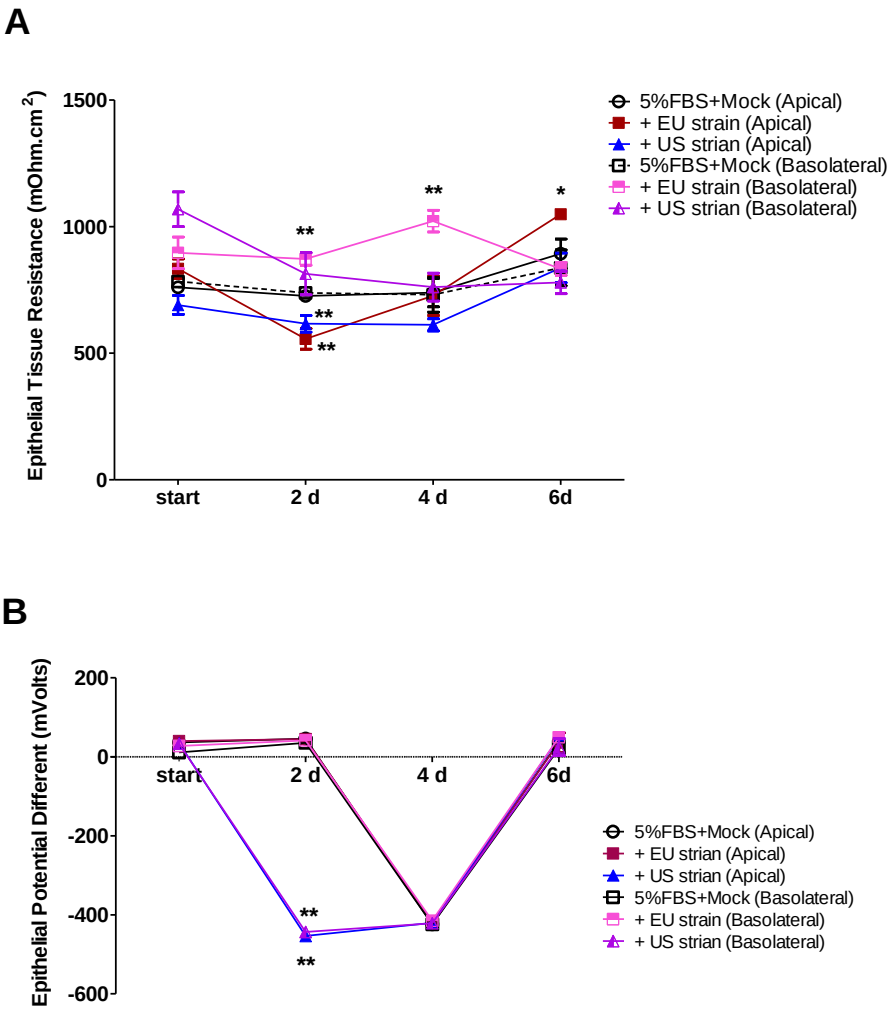

Figure 6 Assessment of endometrial barrier function by the measurement of electrophysiological properties of PE cell by measuring of (A) transepithelial electrical resistance (TER; mOhm.cm^2) and (B) potential differences (PD, mVolts) in PE cell inoculation with PRRS EU strain or US strain at the apical or basolateral site. Data were presented in mean±SEM of TER or PD at 0, 2, 4 or 6 days of infection point (n = 6 experiments in each group). Statistical analysis was performed by ANOVA followed by Dunnett's test compared to control of each group, 5%+Mock (apical) or (basolateral). * and ** represent $p<0.05$ and $p<0.01$ that are significant difference from the control group.

Determination of PRRSV mediators mRNA expression

PE cells showed susceptibility to PRRSV infection. We performed qPCR to determine of PRRSV mediator mRNA expressions in PE cells at 4 dpi (Figure 7). There were up-regulation of both *CD151* and *CD163* mRNA expression in PE cells infected with either EU or US strains compared to mock infection (*P<0.05*).

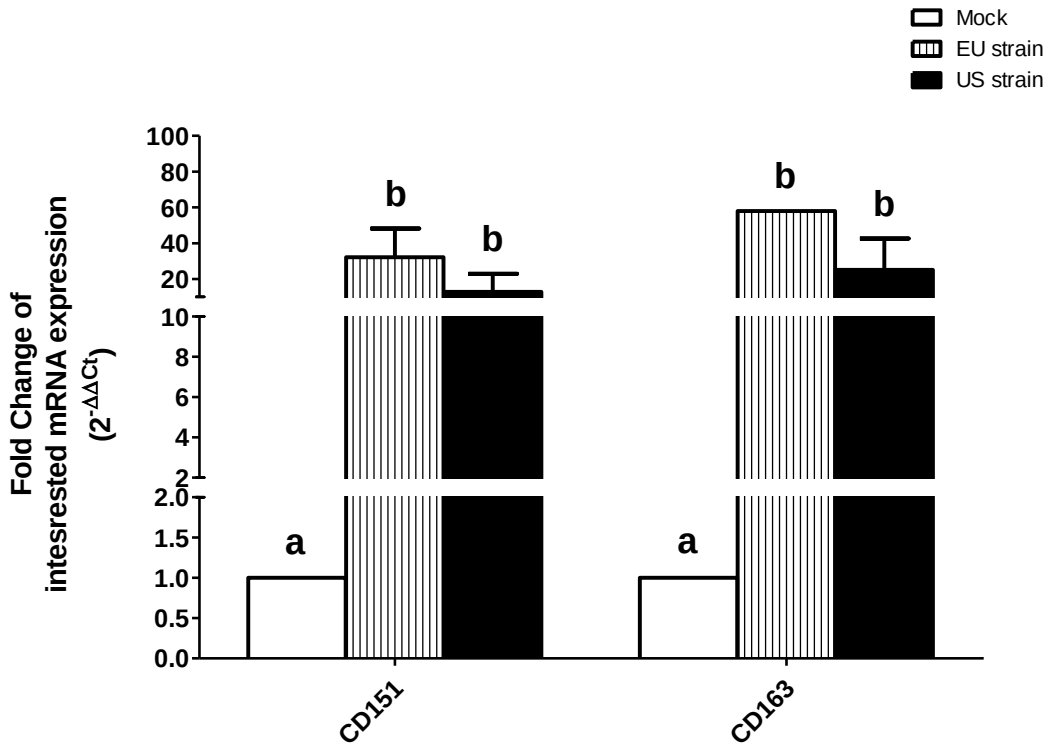

Figure 7 Fold changes of *CD151* and *CD163*, the specific PRRSV receptor mRNA expression in response to PRRS viral infection at **(A)** the apical site or **(B)** the basolateral site. Cell lysated RNA collected from the inserted filter after 4 days of PRRSV inoculation was assayed for qRT-PCR. Data was represented in means±S.E.M of fold changes ($2^{-\Delta\Delta Ct}$) from house-keeping gene, *GAPDH* mRNA expression. Experiments were conducted in triplicate. Bars with different letters are significantly different at the value of $p<0.05$ analyzed by one-way ANOVA and Newmans-Keul post-hoc comparison at each site of infection.

Determination of PRRSV mediators cellular protein expression

According to mRNA expressions of PRRSV mediators, immunohistochemistry was performed to observed cellular expressions of PRRSV mediators in PE cells during 2-6 dpi. As figure 8 shown, PRRSV apical infections were markedly changed PRRSV mediator expressions. Respectively, there were up-regulations of CD163 and Sn (CD169) expressions by US strain infection since 2-4 dpi (*P<0.05*). EU strain infection also increased Sn (CD169)

expression in PE cells during 2-4 dpi (*P<0.05*). The expressions of Sn (CD169) were up-regulated from 2 to 6 dpi (*P<0.05*). CD163 were decreased expressions from 4-6 dpi by US and EU strain infection (Figure 8 and 9) (*P<0.05*).

There were some changes in PRRSV mediator expressions by PRRSV basolateral infection. US strain infection increased Sn (CD169) expression during 2-6 dpi (P<0.05), whereas EU strain up-regulated Sn (CD169) expressions at 4 dpi (Figure 10 and 11) (P<0.05). There were down-regulations of CD163 expressions modified by PRRSV infection from 4-6 dpi (*P<0.05*). PRRSV basolateral infections did not change the CD163 at 2 dpi (Figure 8B and 9B) (P<0.05).

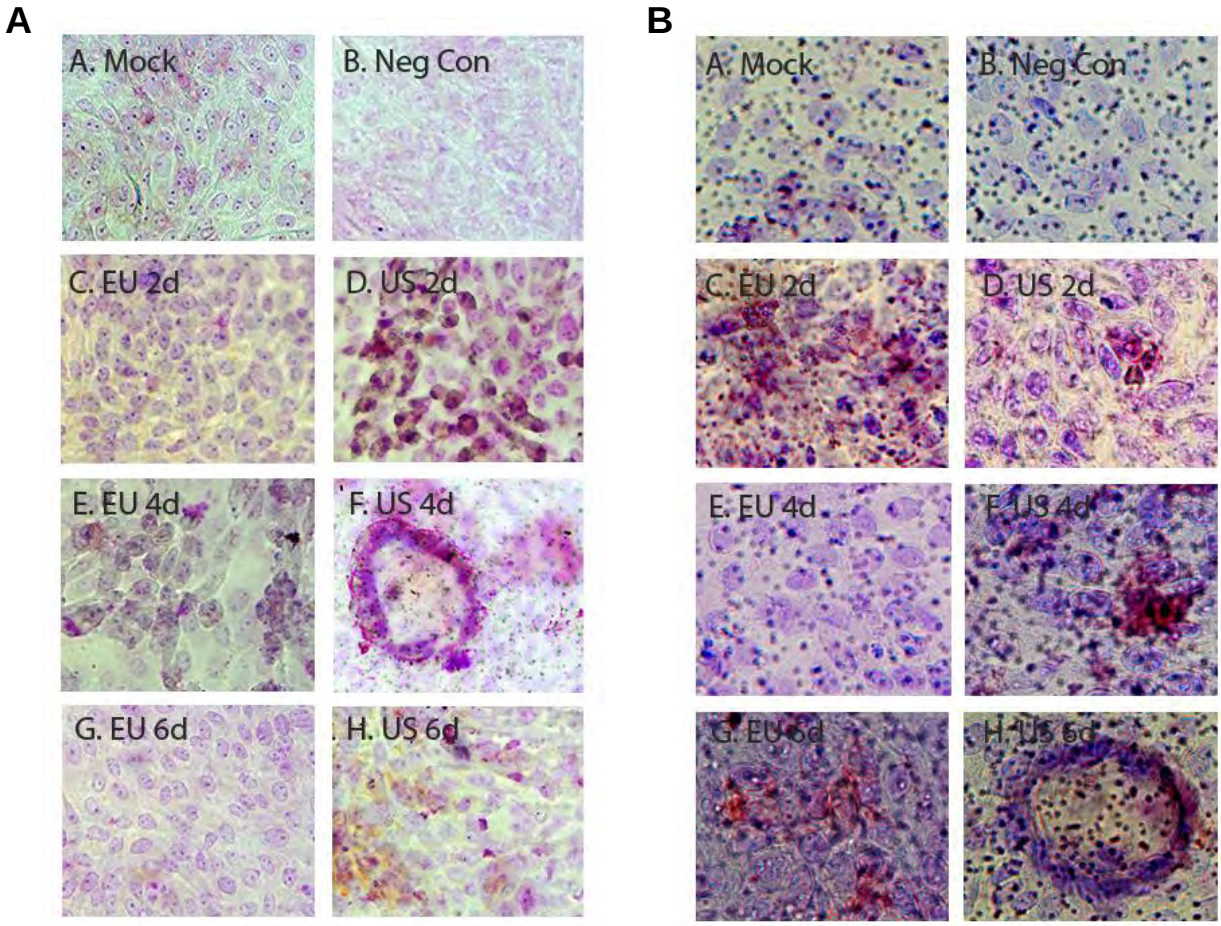

Figure 8 Immunofluorescence assay for CD163 detection. PE cultured on inserted cell filter were inoculated with PRRSV strain EU or US at (A) apical or (B) basolateral side. Cells were then stained with rabbit polyclonal anti-CD163 antibody at 2 (2d), 4 (4d) or 6 (6d) days post infection. This figure shows the positive immunoreactive results (dark brown) in the representative images of three independent experiments. Magnification, 200×.

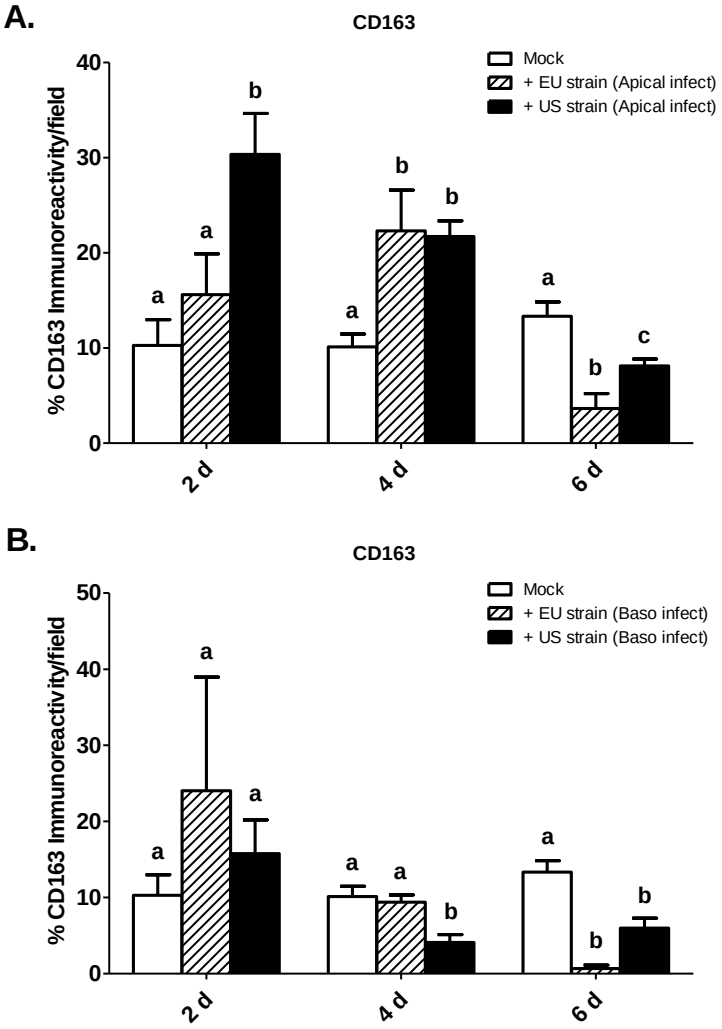

Figure 9 Effect of PRRSV infection on CD163 expression. PE cultured on inserted cell filter were inoculated with PRRSV strain EU or US at (A) apical or (B) basolateral side. Cells were then stained with rabbit polyclonal anti-CD163 antibody at 2 (2d), 4 (4d) or 6 (6d) days post infection. Histogram represents means ± SEM of a percent of the immunoreactive cells to the total cells. Bars with different letters are significantly different at the value of $p<0.05$ analyzed by one-way ANOVA and Newmans-Keul post-hoc comparison at each time of infection.

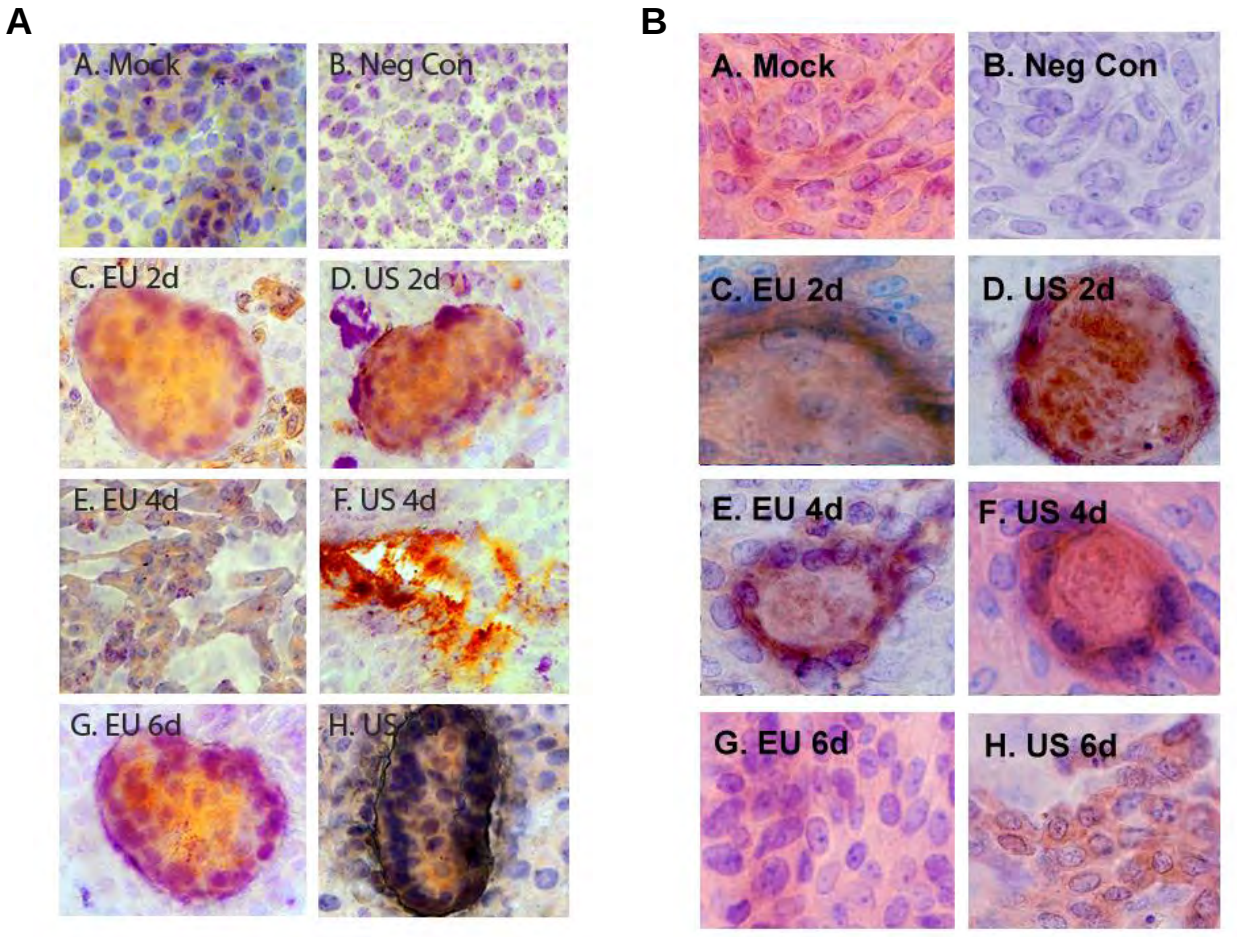

Figure 10 Immunofluorescence assay for siaoadhesin/CD169 (Sn/CD169) detection. PE cultured on inserted cell filter were inoculated with PRRSV strain EU or US at (A) apical or (B) basolateral side. Cells were then stained with rabbit polyclonal anti- Sn/CD169 antibody at 2 (2d), 4 (4d) or 6 (6d) days post infection. This figure shows the positive immunoreactive results (dark brown) in the representative images of three independent experiments. Magnification, 200×.

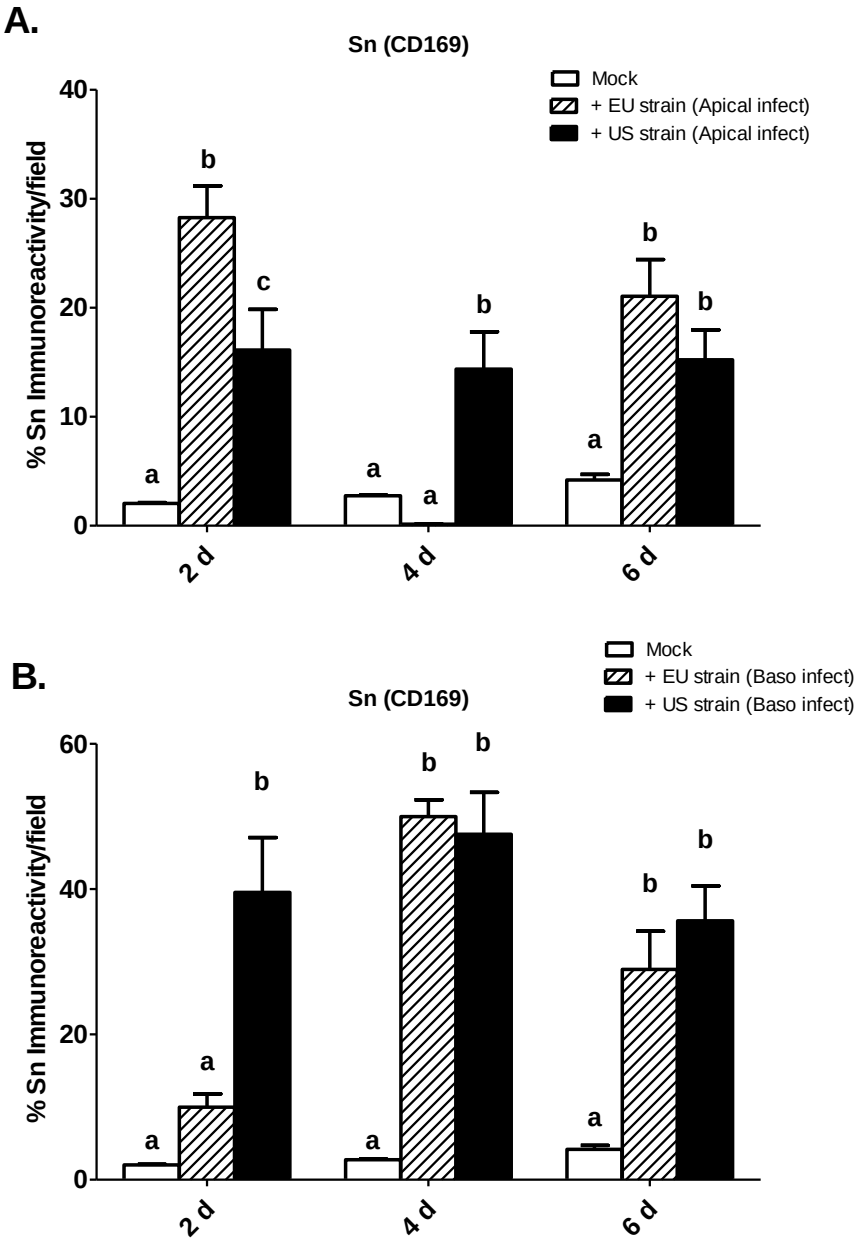

Figure 11 Effect of PRRSV infection on siaoadhesin/CD169(Sn/CD169). PE cultured on the inserted cell filter were inoculated with PRRSV strain EU or US at (A) apical or (B) basolateral side. Cells were then stained with rabbit polyclonal anti-Sn/CD169 antibody at 2 (2d), 4 (4d) or 6 (6d) days post infection. Histogram represents means ± SEM of a percent of the immunoreactive cells to the total cells. Bars with different letters are significantly different at the value of $p<0.05$ analyzed by one-way ANOVA and Newmans-Keul post-hoc comparison at each time of infection.

DISCUSSION

PRRSV infection has been characterized by causing reproductive failure in breeder and respiratory failure in growing pigs. The 2 genotypes of PRRSV, EU and US, cause similar clinical severity of reproductive failure (4). Due to the cellular tropism of PPRSV, its infection is limited to some kinds of cells. The natural targets of PRRSV infection are macrophage and monocyte lineages (5, 6). CL2621, MA-104, and MARC-145 cell lines have been reported to PRRSV susceptible (4, 7, 8). Recently, porcine endometrial endothelial cell line has been generated and demonstrated PRRSV susceptibility (4). Susceptibility to PRRSV infection is determined by the presence or absence of specific PRRSV entry mediators in the target cells. Many molecules have been described as specific mediators, including Sn, CD163, integrin and vimentin. There has been no reported about the susceptibility of PRRSV in PE cells. Therefore, our study demonstrated PRRSV mediator expressions in PE cells to investigate the susceptibility of PRRSV infection in the reproductive system. In the present study, PRRSV protein expression can be observed in PE cells infected with either EU or US strains. Thus, PE cells are also susceptible to PRRSV infection. To compare two sides of infection, apical and basolateral infection was performed. Basolateral infection simulated PRRSV infection between blood circulation to endometrial cell, and apical infection referred to PRRSV infection from fetus to dam. In PE cells, apical PRRSV infection increased PRRSV expression higher than basolateral infection. Therefore, PRRSV infection in maternal endometrial cell might favor PRRSV infection from fetus rather than from dam's circulation.

In this study, some of PRRSV mediators were expressed in PE cells at basal condition. Infection with PRRSV modulated PRRSV mediator either mRNA and protein expressions. To initially determine the presence of PRRSV mediators, we observed mRNA expression of PRRSV mediators only apical PRRSV infection at 4 dpi, according to the cytopathic changes by PRRSV infection. Changes in mRNA expressions of CD163, Sn and integrin indicated the target genes of PRRSV infection in PE cells. During 2-4 dpi, protein expression of CD163 and Sn were changed in accordance with mRNA expression by PRRSV apical infection. It could be confirmed that CD163 and Sn related to PRRSV infection in PE cells. Up-regulation of CD163 and Sn expressions by PRRSV apical infection indicated that PRRSV re-infection from fetus to maternal endometrium might facilitate PRRSV re-infection. At 6 dpi, many mediators were decreased and/or unchanged, may be due to non-viable PE cells caused by PRRSV infection at 6 dpi. The non-viable cells can be observed by

hematoxillin negative staining. PRRSV infection might progressively destroy PE cells depended on time. In addition, PE cells might become aging and degeneration.

CD151 is one of tetraspanin family members. Most of these members are cell-surface proteins characterized by the presence of four hydrophobic domains. They play roles in the regulation of cell development, activation, growth, and motility by mediating signal transduction events. In humans, it is known as a promoter of metastasis of cancer cells (9–11). Evidence showed that CD151 confers susceptibility to PRRSV infection. CD151 was expressed in all susceptible cell lines including MA-104, MARC-145, COS-7, and Vero cells, which are derived from African green monkey kidney. However, it was not expressed in BHK-21 and MDBK cells derived from the kidneys of the other species. Transfection of a CD151 expressing clone into BHK-21 cells changed the susceptibility to PRRSV (12). PE cells in the present study revealed the mRNA expression of CD151. Its protein expression could not be shown, since the commercial antibody for tested has not yet been available. However, up-regulation of CD151 mRNA expression over 20 folds was found after first infection with PRRSV. The increased CD151 induced by PRRSV infection may lead host susceptible to the following PRRSV infection or cause the cell overgrowth as seen in the histological changes.

CD163, scavenger receptor, is a type 1 transmembrane protein expressed on macrophage and monocyte (13). CD163 functions by binding and internalizing hemoglobin-haptoglobin (Hb-Hp) complexed to protect tissue from oxidative damage (14-16). Although CD163 expression is restricted to macrophage and monocyte. Our study has shown that PE cells also possess this molecule. A recent study has shown that CD163 is essential for PRRSV infection into macrophage (17). After internalisation, CD163 uncoats and releases PRRSV into intracellular component (18, 19). Expressions of CD163 are not only limited to endosome, but also observed in soluble form. CD163 is suggested that it also bind and internalize the virus (20). During this process, CD163 interacts with GP2 and GP4 of PRRSV (20). Nevertheless, the functions of CD163 in PE cells have not been identified. The up-regulation of CD163 in PE cells by PRRSV infection might be related to the enhanced PRRSV susceptibility of the cells. There has been reported that CD163 expression can be regulated by pro-inflammatory and anti-inflammatory mediators such as lipopolysaccharide, IFN- γ, TNF-α, interleukin (IL) 6 and IL10 (21). After PRRSV infection, change in CD163 expression in PE cells might be mediated via such cytokines. Interaction between Hb-Hp complex and CD163 induced IL-10 secretion in CD163-bearing cells, which indicated the

functional role of CD163 an innate immune response (22). Up-regulation of IL-10 gene expression was observed in porcine peripheral blood mononuclear cells (23). PRRSV infection in PE cells may modulate cellular immune response via mediating CD163 expression. Thus, this suggestion is important for further study.

Sn (CD169 or Sialec-1) is a type I transmembrane glycoprotein consisting of extracellular Ig-like domains and a short cytoplasmic tail (24). The extracellular domain functions by binding sialic acid expressed on pathogen glycoprotein (25). Sn expression is highly regulated and restricted to tissue macrophages and secondary lymphoid tissues (26). The expression in macrophage is related to facilitate sialylated pathogen interaction, such as PRRSV internalization (27). The interaction between PRRSV and Sn occurs via binding of sialic acids on GP5 and Sn (28). In this study, Sn expressions were observed in PE cell whether it was infected with PRRSV or not. PE cells were highly up-regulated Sn mRNA and protein expression after infection with PRRSV EU and US strains. This can be indicated that Sn is the highly sensitive molecule in PE cells depending on PRRSV infection. Previous study demonstrated that Sn joined forces with CD163 interacted with PRRSV. It is possible that Sn acted as an initial attaching molecule with PRRSV and mediating internalisation, then CD163 played a role for uncoating during the later step (2). Expression both of CD163 and Sn resulted in highly susceptible to PPRSV infection of the target cells comparing with cell expression only Sn or CD163 (2). PE cells infected with PRRSV were increased both Sn and CD163 expression. Therefore, PE cells infected with PRRSV became more susceptible to subsequent PRRSV infection. Sn expression can be induced by IFN-$\propto$ and IFN- γ in monocytes and macrophages (29). PRRSV infection stimulated IFN- γ in the target cells, which is a concordance to our preliminary result in PE cells (unpublished data). This may result in up-regulation of Sn expression in the PE cells.

At the present, utilization of PRRSV mediators between different PRRSV strains have not been fully elucidated. Our result also compared the different mediator expression between EU and US strains. The present study indicated that PE cells can be infected with both of two strains. Although, either apical and basolateral infection with US strain infection elicited higher PRRSV expressions in the target cells than EU strain. Both of two strains mediated the similar mediators, particular Sn and CD151/163, but different degree. Infection with US strain up-regulated CD151/163 and Sn earlier than EU strain since 2 dpi. Therefore, PRRSV infection in PE cells with US strain was more virulent than EU strain.

Non-permissive cell line became fully permissive for PRRSV when gene transfected with CD163 alone (18). In addition, mAb specific for Sn did not block PRRSV infection in MARC-145, but blocked the infection in pulmonary alveolar macrophage (PAM) (30). Thus, CD163 was suggested to be a core PRRSV receptor, whereas Sn is an accessory. Not all PRRSV permissive cells required only CD163 expression. Binding assay demonstrated that many non-permissive cell required more than one receptor for PRRSV internalization (31,32).

In the focus of innate immune response to PRRSV infection, PE cells are firstly revealed in the present study to secrete some pro-inflammatory cytokines, i.e. IL-1, IL-6, IL-8, TNF-α and IFN-γ at low concentration. However, IL-8 which is the chemokines secreted by many epithelial cells was gradually secreted in the highest concentration over time. It suggested that the PE cells alone have the competency to protect itself from the pathogens. PRRSV can induce the increased IL-6, IL-8 or IFN-γ cytokine production and secretion as seen in the macrophages, which is the target cells of PRRSV (33). The evidence of PE secreted cytokine response to PRRSV may reflect the function of PRRSV receptors, CD163 or Sn expressed by PE cells. In addition, the highest cytokine production was shown at 6 dpi consistent to the highest level of PRRSV receptor expression in the US strain infected-PE cells. The increased IL-8 and IFN-γ cytokine responses has been suggested to be a good prognosis for viral clearance. The non-PRRSV persistent pigs in the recent study had higher the IL-8 secretion than PRRSV-persistent pigs (34). It draws your attention that the inhibition of IL-8 and IFN- γ secreted to the basolateral side when EU strain and US strain PRRSV respectively was infected via the basolateral sides. It implied the PRRSV carried out in the blood circulatory system may cause the problem of PRRSV persistent in the pigs. In addition, the tissue disturbance or immunopathology caused by the consequence of the inflammatory process may be occurred.

CONCLUSION

Mechanisms of PRRSV infection mediated via specific mediators to permit viral attachment, internalization and uncoating. Sn and CD151/163 play together to internalize and uncoating PRRSV. Form our result, PE cell is one of the PRRSV permissive cell,since PE cells expressions of CD151/163 and SN. Due to the modulation of PRRSV mediators, infection in PE cells with US strain is more virulent than EU strain. In PE cell, apical PRRSV infection is more favored, which is increased cellular susceptibility for subsequent infection. PRRSV mediator in PE cells may become the targets for blocking the horizontal transmission

by anti-viral reagent. In addition, expressions of PRRSV mediators may be useful for isolating the viruses and developing more potential vaccines.

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