PHYSIOLOGICAL AND IMMUNOLOGICAL RESPONSES OF PORCINE PRIMARY ENDOMETRIAL CELLS TO PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME (PRRS) VIRUS INFECTION

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การตอบสนองทางสรีรวิทยาและภูมิคุ้มกันของเซลล์ปฐมภูมิเยื่อบุมดลูกสุกรต่อการติดเชื้อไวรัสพีอาร์อาร์เอส

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Thesis Title	PHYSIOLOGICAL AND IMMUNOLOGICAL RESPONSES OF PORCINE PRIMARY ENDOMETRIAL CELLS TO PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME (PRRS) VIRUS INFECTION
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มัธย์ฤน โล่ทอง : การตอบสนองทางสรีรวิทยาและภูมิคุ้มกันของเซลล์ปฐมภูมิเยื่อบุมดลูกสุกรต่อการติดเชื้อไวรัสพีอาร์อาร์เอส. (PHYSIOLOGICAL AND IMMUNOLOGICAL RESPONSES OF PORCINE PRIMARY ENDOMETRIAL CELLS TO PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME (PRRS) VIRUS INFECTION) อ.ที่ปรึกษาหลัก : รศ. สพ.ญ. ดร.สุทธาสินี ปุญญโชติ, อ.ที่ปรึกษาร่วม : รศ. ดร.ฉัตรศรี เดชะปัญญา,อ. น.สพ. ดร.สุพจน์ วัฒนะพันธ์ศักดิ์

การติดเชื้อไวรัสพีอาร์อาร์เอสถูกจำกัดเป็นอย่างยิ่งต่อเซลล์ที่มีการแสดงออกของตัวรับของไวรัสเท่านั้น อวัยวะในระบบสืบพันธุ์มีการแสดงออกของอาการทางคลินิกที่จำเพาะต่อการติดเชื้อพีอาร์อาร์เอสอาจเป็นบริเวณที่มีความสำคัญของปัญหานี้ การติดเชื้อที่คงอยู่ภายในฝูงก่อให้เกิดการติดเชื้อซ้ำไม่ว่าจะเกิดจากการแพร่ระหว่างตัวสุกรหรือระหว่างแม่สู่ลูกซึ่งไม่สามารถกำจัดให้หมดไปจากฝูงได้ การศึกษาครั้งนี้ตรวจสอบความเป็นไปได้ของเซลล์เยื่อบุมดลูกสุกรต่อการไวรับการติดเชื้อไวรัสพีอาร์อาร์เอสซึ่งเป็นสาเหตุปฐมภูมิต่อการติดเชื้อแบบเรื้อรัง โดยประเมินความไวรับของเซลล์เยื่อบุมดลูกสุกรต่อการติดเชื้อไวรัสพีอาร์อาร์เอสจากการตอบสนองทางเซลล์และระบบภูมิคุ้มกันที่เกี่ยวข้องกับการเพิ่มจำนวนของไวรัส การแพร่กระจายของไวรัส และการหมุนเวียนของไวรัส ซึ่งมีการเปรียบเทียบผลที่แตกต่างกันซึ่งเกิดจาก การติดเชื้อไวรัสต่างสายพันธุ์ (สายพันธุ์ I และ II) หรือ การติดเชื้อต่างทิศทาง (ฝั่งบนของเยื่อบุ และ ฝั่งฐานของเยื่อบุ) เซลล์เยื่อบุมดลูกสุกรชนิดปฐมภูมิ (เซลล์พีอี) ถูกแยกจากมดลูกของสุกรอายุ 4-6 เดือนที่ปลอดเชื้อไวรัสพีอาร์อาร์เอส (จำนวนตัวอย่างสุกร = 5 ตัว) และถูกเพาะเลี้ยงในอาหารเลี้ยงเชื้อมาตราฐานที่มีซีรัมลูกวัวร้อยละ 5 เมื่อเซลล์โตเต็มภาชนะจึงนำมาอาบทางฝั่งบนของเซลล์ หรือฝั่งฐาน ด้วยไวรัสพีอาร์อาร์เอสทั้ง 2 สายพันธุ์ที่ถูกแยกจากปอดที่ติดเชื้อ และทำการบ่มเพาะเป็นเวลา 1 ชั่วโมง มีการประเมินการเกิดความผิดปกติของเซลล์ (CPE) ทุกวัน จำนวนเซลล์ที่มีไวรัสพีอาร์อาร์เอสและการแสดงออกของโปรตีนที่เป็นตัวรับของไวรัสพีอาร์อาร์เอส ได้แก่ CD151 CD163 sialoadhesin (Sn) integrin และ vimentin ถูกตรวจสอบด้วยวิธีอิมมูโนฮีสโตเคมมิสทรี และการขับหลั่งของไซโตคายน์ที่เกี่ยวข้อง ได้แก่ CCL2 IL-1β IL-6 IL-8 IFN-ɣ และ TNF-α ถูกประเมินด้วยวิธี enzyme linked immunosorbent assay (อีไลซ่า) ณ วันที่ 0 2 4 และ 6 ของการติดเชื้อ การเปลี่ยนแปลงของยีนของตัวรับเชื้อไวรัสพีอาร์อาร์เอส ตัวรับ toll-like และไซโตคายน์ที่เกี่ยวข้องถูกประเมินโดย real-time RT-PCR ผลของการติดเชื้อซ้ำโดยไวรัสพีอาร์อาร์เอสต่อการเปลี่ยนแปลงจากการติดเชื้อครั้งแรกถูกประเมินโดยทำการอาบไวรัสซ้ำต่อเซลล์พีอี ณ วันที่ 4 ของการติดเชื้อครั้งแรก ในช่วงต้นของการติดเชื้อ ณ วันที่ 4 ของการติดเชื้อ การเกิด CPE และโปรตีนของเชื้อไวรัสพีอาร์อาร์เอสสามารถพบได้ในเซลล์พีอีที่ติดเชื้อจากฝั่งบนของเซลล์ แต่หลังจากนั้นต่อมาจึงสามารถพบได้ในเซลล์ที่ติดเชื้อจากฝั่งฐาน การติดเชื้อไวรัสายสายพันธุ์ II ก่อให้เกิดผลของการติดเชื้อได้มากกว่าสายพันธุ์ I (*p<0.05*). เซลล์พีอีมีการแสดงออกของยีนตัวรับไวรัสพีอาร์อาร์เอสโดยสามารถพบได้ก่อนติดเชื้อ ได้แก่ CD151 Sn integrin และ vimentin ยกเว้น CD163 ไวรัสสายพันธุ์ I เพิ่มการแสดงออกของ *CD151 CD163 Sn* และ *integrin* มากกว่าเซลล์ที่ไม่ได้อาบไวรัสและเซลล์ที่อาบไวรัสสายพันธุ์ II *(p<0.05)* พบการเปลี่ยนแปลงที่แตกต่างกันของโปรตีนตัวรับไวรัสพีอาร์อาร์เอสระหว่าวันที่ 4-6 ของการติดเชื้อ การอาบไวรัสสายพันธุ์ I ฝั่งบนของเซลล์เพิ่มโปรตีนตัวรับเชื้อไวรัสทุกตัวยกเว้น Sn *(p<0.05)* แต่ที่ฝั่งบนการอาบด้วยไวรัสสายพันธุ์ II ลดโปรตีน Sn, integrin และ vimentin *(p<0.05)* การอาบไวรัสที่ฝั่งฐานด้วยสายพันธุ์ I และ II ลดจำนวนโปรตีน integrin และ vimentin *(p<0.05)* แต่กลับเพิ่มโปรตีน CD151, CD163 และ Sn *(P<0.05)* การเพิ่มการแสดงออกของยีน *TLR1/TLR3* และ*TLR10* ถูกเหนี่ยวนำโดยการติดเชื้อครั้งแรกโดยไวรัสสายพันธุ์ I และ II ตามลำดับ การติดเชื้อโดยไวรัสสายพันธุ์ I และ II ลดการแสดงออกของยีน *TLR4 (p<0.05)* การติดเชื้อซ้ำโดยเฉพาะไวรัสพีอาร์อาร์เอสสายพันธุ์ I เพิ่มการแสดงออกของยีน *TLR1 TLR2* และ *TLR4 (p<0.05)* หลังจากนั้นต่อมาการลดลงของยีน *TLR5* และ *TLR8* สามารถพบได้โดยไวรัสทั้ง *2* สายพันธุ์ *(p<0.05)* การติดเชื้อซ้ำโดยไวรัสสายพันธุ์ I และ II ลดการแสดงออกของยีน *IL-6* อย่างสมบูรณ์ แต่ไม่มีผลต่อยีนไซโตคายน์ชนิดอื่น การติดเชื้อปฐมภูมิไวรัสพีอาร์อาร์เอสไม่เปลี่ยนแปลง การขับหลั่งของ CCL2 IL1β IL-8 และ IFN-ɣ *(p>0.05)* ณ วันที่ 6 หลังการติดเชื้อการขับหลั่งของ IL-6 เพิ่มขึ้นโดยไวรัสสายพันธุ์ I จากการติดเชื้อครั้งแรกทั้งทางฝั่งบนของเซลล์หรือฝั่งฐาน *(p<0.05)* การติดเชื้อปฐมภูมิและการติดเชื้อซ้ำลดการขับหลั่งของ TNF-α อย่างมีนัยสำคัญ *(p<0.05)* เป็นที่น่าสังเกตว่าสารละลายที่เก็บจากทุกเซลล์ที่ได้รับการติดเชื้อมีการติดเชื้อของไวรัสพีอาร์อาร์เอสในปริมาณ $TCID_{100}$ ต่อ 1 มิลลิลิตร ตลอดระยะเวลาการศึกษา จากการทดลองนี้สรุปได้ว่าเซลล์เยื่อบุมดลูกมีความไวรับต่อการติดเชื้อไวรัสพีอาร์อาร์เอสได้ทั้งทางฝั่งบนและทางฝั่งฐานของเซลล์และมีการติดเชื้อซ้ำหมุนเวียนเป็นเวลานาน ความไวรับต่อการติดเชื้อไวรัสพีอาร์อาร์เอสอาจเกิดจากการเกิดปฏิสัมพันธ์ระหว่างไวรัสพีอาร์อาร์เอสต่อตัวรับ TLRs หรือตัวรับของไวรัสเอง โดยการเปลี่ยนแปลง TLR ตัวรับของไวรัส และการตอบสนองของไซโตคายน์ที่เพิ่มขึ้นหรือลดลงนั้นขึ้นอยู่กับสายพันธุ์ของไวรัสและทิศทางของการติดเชื้อ ไวรัสพีอาร์อาร์เอสสายพันธุ์ II มีความรุนแรงต่อการทำลายเซลล์มากกว่าสายพันธุ์ I แต่สายพันธุ์ I มีผลกระทบต่อความไวรับต่อการติดเชื้อครั้งถัดไป ดังนั้นการติดเชื้อโดยตรงจากไวรัสพีอาร์อาร์เอสต่อเซลล์เยื่อบุมดลูกสุกรแบบปฐมภูมิอาจมีบทบาทสำคัญต่อการเกิดความล้มเหลวของระบบสืบพันธุ์และอาจเป็นสาเหตุของการติดเชื้อแบบถาวรในแม่สุกร

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Porcine reproductive and respiratory syndrome virus (PRRSV) is highly limited to only cell subsets that express PRRSV receptors. Reproductive organ revealing the typical signs of PRRSV infection may be the critical site of problem syndromes. Persistent PRRSV producing re-infection via horizontal or vertical transmission could not be eradicated from herds. This research examined the possibility of porcine endometrium to be a PRRSV permissive cell and serves as the primary cause of the persistent PRRSV. Cellular and immunological in response to PRRSV relevant to viral replication, shedding and re-circulation was assessed. The different outcomes between the different genotypes (type I vs. II), and routes of infection (apical vs. basolateral) were compared. Porcine glandular endometrial epithelial cells (PE) isolated from 4-6 months old PRRSV-free pre-puberty gilts (n=5 pigs) were cultured in standard medium DMEM with 5% fetal bovine serum until 90% confluent. Fresh isolated PRRSV type I or type II (at $TCID_{100}$/2 ml) were inoculated to apical or basolateral membrane of PE for 1 hr. The occurrence of cytopathic effect (CPE) were observed daily. PRRSV-positive cells and cellular PRRSV mediator proteins, CD151, CD163, sialoadhesin (Sn), integrin and vimentin, were quantified by immunohistochemistry (IHC). Related cytokine secretion, CCL2, IL-1β, IL-6, IL-8, IFN-g and TNF-α was measured by enzyme linked immunosorbent assay (ELISA), at 0, 2, 4 and 6 day-post-infection (dpi). The mRNA expression of PRRSV mediator, toll-like receptors (TLRs) and cytokines were evaluated by real-time RT-PCR. Effects of PRRSV re-infection in modulating all the responses of primary infection were considered by repeating the infection at 4 dpi at the same PE. At early stage of infection, at 4dpi, CPE along and PRRSV proteins was observed in apical PRRSV infected PE, but was observed later in basolateral-infected PE. Infection with type II produced these infectivity effects rather than type I *(p<0.05).* Prior to infection, mediator proteins CD151, Sn, integrin and vimentin but not CD163 were expressed. Type I up-regulated *CD151*, *CD163*, *Sn* and *integrin* mRNA higher than mock and type II *(p<0.05).* Changes of mediator proteins were observed differently during 4-6 dpi *(p<0.05).* Apical infection with type I up-regulated all mediators except Sn, whereas type II down-regulated Sn, integrin and vimentin. Basolateral type I and II infection down-regulated integrin and vimentin *(p<0.05),* but up-regulated CD151, CD163 and Sn *(p<0.05).* Up-regulation of *TLR1/TLR3* and *TLR10* were induced by primary infection with type I and II, respectively. All primary infection down-regulated *TLR4* mRNA *(p<0.05).* Re-infection with PRRSV particularly type I up-regulated the level *of TLR1*, *TLR2* and *TLR4* expression *(p<0.05).* Down-regulation of *TLR5* and *TLR8* were later observed in primary or re-infected PE cells *(p<0.05).* Re-infection with type I or II completely decreased *IL-6* mRNA, but not other genes. Primary PRRSV infection could not alter CCL2, IL1β, IL-8 and IFN-g secreted by PE , but type I infection increased IL-6 secretion (*p<0.05).* Primary or re-infection with PRRSV type I or II dampened TNF-α secretion significantly *(p<0.05).* Noticeably, in the present study, supernatant collected from all PRRSV-infected cells contains PRRSV at $TCID_{100}$/ml throughout the study. In summary, endometrial cells are susceptible to either apical and basolateral PRRSV infection, and long-lasting re-circulate PRRSV. Effects of primary infection may be mediated by TLRs or mediators. Modification in the synthesis of TLRs, PRRSV mediators and cytokines by PRRSV could be enhanced or suppressed depending on time course, genotype or route of infection. PRRSV type II has more virulence than type I but PRRSV type I produced more to susceptibility for executive infection. Therefore, direct PRRSV infection to PE cells may play a role in PRRSV-induced reproductive failure and may be the cause of persistent PRRSV infection in sow.

Field of Study:	Animal Physiology	Student's Signature .....
Academic Year:	2018	Advisor's Signature .....
		Co-advisor's Signature .....
		Co-advisor's Signature .....

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LIST OF ABBREVIATIONS

Δ	change
°C	centigrade
µg	microgram
µl	microliter
ANOVA	analysis of variances
BHK-21	baby hamster kidney cell line
BSA	bovine serum albumin
CAMs	cellular adhesion molecules
CCL	chemokine (C-C motif) ligand
$CD4^+$	helper T cell
$CD8^+$	cytotoxic T cell
CD163	cluster of differentiate 163
cDNA	complementary deoxyribonucleic acid
cm^2	square centimeter
CPE	cytopathic effect
C_t	threshold cycle
CXCL	chemokine (C-X-C motif) ligand

DAMP	damage-associated molecular patterns
DEPC	diethylpyrocarbonate
DMEM	Dulbecco's Modified Eagle Medium
DNA	deoxyribonucleic acid
dpi	day post infection
E	viral envelope protein
E_2	estrogen
EAV	equine arteritis virus
ELISA	enzyme linked immunosorbent assay
FBS	fetal bovine serum
FGF	fibroblast growth factor
Fig	figure
g	gram
g	relative centrifugal force
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
G-CSF	Granulocyte colony-stimulating factor
GE	glandular endometrial epithelium
GM-CSF	Granulocyte macrophage colony-stimulating factor

GP	viral glycoprotein
HEC-1-A	human endometrial cell line
HIV	human immunodeficiency virus
hr	hour
HRP-conjugated	horse-radish peroxidase conjugated
IFN	interferon
Ig	Immunoglobulin
IL	interleukin
IRF-3	interferon regulatory transcription factor
L-DCs	lung dendritic cells
LDV	lactate dehydrogenase-elevating virus
LE	luminal endometrial epithelium
LIF	leukemia inhibiting factor
LPS	lipoloplysaccharide
M	viral membrane protein
MA-104	African Green monkey kidney cell line
MARC-145	Derivative of MA-104 cell line
MAPK	mitogen-activated protein
MCP	monocyte chemoattractant protein

min	minutes
MIP	macrophage inflammatory protein
mm^2	square millimeter
MoDCs	monocyte-derived dendritic cells
mRNA	messenger ribonucleic acid
MyD88	Myeloid differentiation primary response 88
NK cell	natural killer cell
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
nm	nanometer
nsp	viral non-structural protein
OD	optical density
ORF	open-reading frame
P_4	progesterone
PAMPs	pathogen-associated molecular patterns
PBS	phosphate buffer saline
PBST	phosphate buffer saline with tween
PDGF	platelet-derived growth factor

PE	primary porcine endometrial cell
PEE	porcine endometrial endothelial cell
PGE-2	Prostaglandin-E2
PGF-2α	Prostaglandin-2α
pp1a	polyprotein 1a
pp1ab	polyprotein 1ab
PRRS	porcine reproductive and respiratory syndrome
PRRSV	porcine reproductive and respiratory syndrome virus
qRT-PCR	reverse transcriptase realtime-polymerase chain reaction
RL95-2	human endometrial cell line
RNA	ribonucleic acid
sec	seconds
Sn (CD169)	sialoadhesin
T25 flask	25 cm^2 flask
$TCID_{100}$	tissue culture infection dose
Th	helper T cell
TIR	toll-interleukin receptor domain

TLR	toll-like receptor
TMB	3,3′,5,5′-Tetramethylbenzidine
TNF	tumor necrosis factor
TRIF	TIR-domain-containing adapter-inducing interferon-β
UTR	untranslated region
VEGF	vascular endothelial growth factor

CHAPTER I
IMPORTANCE AND RATIONALE

Porcine reproductive and respiratory syndrome (PRRS) is one of the most economically concerning disease in global swine industries. The etiologic agent is a porcine reproductive and respiratory syndrome virus (PRRSV), a single stranded-RNA virus, family *Arteriviridae*, order Nidovirales, which causes the characteristics of respiratory distress with increasing susceptibility to secondary infection in growing pigs and reproductive failure in pregnant pigs (Benfield et al., 1992; Cavanagh, 1997). Even though PRRSV-infected pigs do not reveal abnormality of the reproductive organs, they produce the weak-born piglets that are generally immunocompromised causing the susceptibility to secondary infection, a respiratory disease complex in particular. They also subsequently turn to be reservoirs leading PRRSV to re-circulate in the herd. Indeed, the transmission of PRRSV usually occurs via direct contact between pigs (horizontal transmission) or another route, such as inhalation or contamination from infected semen. In addition, vertical transmission resulted from shedding of PRRSV between mothers and fetuses during pregnancy was demonstrated, and is so called congenital transmission (Christianson and Joo, 1994). However, infected fetuses or aborted fetuses from PRRSV-positive sows revealed the absence of severe macroscopic lesions in the internal organs suggesting that the fetal death is not the consequence of PRRSV replication in the internal organs (Rossow et al., 1996). Currently, the microscopic lesion, such as inflammation and vasculitis in the endometrium of the PRRSV-positive pregnant gilts, have been shown to correlate with the fetal death. In addition, the characteristic of fetal death in PRRS could not be observed until the late term of gestation. However, the placental tissues have never been indicated for PRRSV infection or replication (Karniychuk et al., 2012). The fetal implantation site (endometrium/fetal placenta) should be in an attention, because it connects maternal and fetal tissues to each other, and very critical for nourishing of the fetus. This evidence leads to consider that **the endometrial/placental cellular properties and functions may be modified by**

the mechanism of cellular and immunological response to PRRSV contamination at the mucosa directly or indirectly from blood circulation on the basolateral site. The adapted endometrium may cause the susceptibility to PRRSV re-infection from infected-fetus, causing the persistent of PRRSV in the endometrium or pathogenicity related to late term abortion or stillbirth. Since prevention of congenital infection may be important for the control of PRRSV eradication. Better understanding of these phenomena may facilitate preventive strategies

In general, the PRRSV infects only specific cell subsets, which is called PRRSV permissive cells. The natural PRRSV tropism cells is restricted to monocytic lineage macrophages, which express cell surface proteins, sialoadhesin (Sn or CD169) and CD163. The viral entry and uncoating process in host cell which allows the virus to replicate or release requires both Sn and CD163 ($Sn^+/CD163^+$). When the process was occurred, it is notified as the susceptibility of cells to virus. Some non-permissive cells were reported to be susceptible to PRRSV following their expression of PRRSV mediators (Lunney et al., 2016). Other cell surface receptor proteins, such as integrin and vimentin were additionally reported to be putative PRRSV receptors. Expression of these PRRSV receptors enhance PRRSV infection by increasing cellular susceptibility to PRRSV (Zhang and Yoo, 2015). Nevertheless, the mechanism by which PRRSV crosses from the endometrium to the fetal placenta has been described through $Sn^+/CD163^+$ endometrial macrophages (Karniychuk et al., 2011). However, endometrium/fetal placental tissues, which have not been suggested for the expression of Sn or CD163, demonstrate the PRRSV positive cells with apoptosis in the late gestation period. Moreover, the number of PRRSV-positive cells were higher in the myometrium/endometrium of PRRSV-attenuated vaccinated than unvaccinated gilts (Karniychuk et al., 2012). The inactivated PPRSV vaccines have been suggested but dissatisfied to prevent conceptus infection (Scortti et al., 2006; Karniychuk et al., 2012). The attenuated PRRSV vaccination for gilts is preferred since it can reduce the number of PRRSV-positive fetuses by lowering pathology and virus replication in the fetal placenta. Unfortunately, attenuated vaccine virus turns to virulence causing the fetal death or transplacental spread of the attenuated vaccine

virus from mother to fetuses has also been reported (Scortti et al., 2006; Karniychuk et al., 2012). It is possible that the endometrium/fetal placental cells are gradually adapted, i.e. increasing the PRRSV receptor expression when pre-exposure with PRRSV from natural or vaccine leading these cells susceptible to PRRSV re-infection. For the control of PRRSV-induced reproductive problems using the attenuated vaccines, both safety and efficacy need to be concerned.

The cellular and immunological responses have been basically suggested during the course of infection in order to clear the virus within the target organs, which depend on rapid activation of the innate immune response (Karniychuk et al., 2011). The mechanism of innate immune system is initiated by the specific recognition of toll like receptors (TLRs) class of the pattern recognition receptors (PRRs) to the specific molecules of pathogen-associated molecular patterns (PAMPs). In the case of viral pathogens, the viral nucleic acids and viral capsid proteins are predictable. The interaction between PAMPs and TLRs activates the transcription factor nuclear factor-κB (NF-κB) or interferon regulatory 3 (IRF-3) leading to triggering and alteration in the pattern of many gene expressions in the cell (Akira, 2006; Sang et al., 2011). Changes in cellular and immunological responses by PRRSV have been extensively studies using respiratory model, particularly in pulmonary alveolar macrophage (PAM) and poly blood mononuclear cells (PBMC). By which the PRRSV infected respiratory model, potent anti-viral cytokines, the type I interferons (IFN) and pro-inflammatory cytokine gene that represent an important part of innate immunity and adaptive immunity are induced (Akira, 2006). In addition, in PRRSV infected PAMs and PBMC, expression of TLRs expression and cytokine secretion were changed (Liu et al., 2009; Zhang et al., 2013; Tu et al., 2015). Recently, studies about host immune response against PRRSV infection using the reproductive model have been examined. Experimental PRRSV inoculation modulated cytokine production in late gestation pigs by increasing systemic production of IFN-α, chemokine ligand (CCL) 2, IFN-γ and tumor-necrosis factor (TNF) α (Rowland, 2010; Ladinig et al., 2014). Modification of TLRs expression and cytokine production or increased activated NFκB by PRRSV may be associated with immunomodulatory response and consequences of target organ disorders. Importantly, up-regulation of interleukin (IL) 10, an immunosuppressive

cytokine, has been suggested to be an important evasion mechanism of PRRSV by modulating host immune responses (Suradhat and Thanawongnuwech, 2003; Suradhat et al., 2003). However, the severity of clinical signs depends upon the route of infection and PRRS viral genotype, suggesting the different cellular and immunological responses (Novakovic et al., 2016).

PRRSV has been genetically categorized into type I (European; EU genotype) and type II (North American; US genotype). Even though they share 55-80% homology of their genetic sequences (Music and Gagnon, 2010), the different genotypes of PRRSV demonstrated the different host immunomodulation due to the variable expressions of the viral proteins (Zhang et al., 2013). PRRSV type II has been reported as a virulent genotype, since it causes more severe respiratory distress than the type I (Scortti et al., 2006). Since unavailability of the comparative studies among EU/US PRRSV in reproductive model, the different severity of reproductive organ disorders caused by the type I and type II remains unclear. The endometrial itself offers the powerful local innate immune responses to pathogens. Thus, it is of interest to exploit the porcine endometrial cell culture model to investigate the mechanism of PRRSV infection and host immune response associated with reproductive organs disorders. Particularly, the viral re-circulation and release from the endometrium and placenta may relate to persistent PRRSV in pigs.

This study determined the mechanism of PRRSV infection locally at the endometrial epithelial cell. The cellular mechanism and immunological response to PRRSV infection relevant to viral replication, viral release and increase of susceptibility to re-infection with PRRSV were examined using porcine primary glandular endometrial epithelial cell culture (PE cells). The comparison between genotypes (type I vs. type II) and between the route of infection (mucosa vs. basolateral side) was also evaluated. The understanding of PRRSV mechanisms modulating the immune response of the host might be a useful target for design of effective control and eradication program for PRRS.

Objectives of Study

The main objectives of this study are

1. To evaluate **the cellular responses** of PE cells to PRRSV infection in the endometrium regarding to the epithelial histology and the modification of PRRSV receptor expression related to viral replication in the different genotypes and routes of infection.
2. To evaluate **the mucosal innate immunological system responses** of PE cells to PRRSV infection in the endometrium regarding to TLRs expression and function releasing the related cytokines in the different genotypes and routes of infection.
3. To evaluate **the susceptibility of PRRSV re-infection** in the PE cells, primary infected with PRRSV regarding to the cellular responses and the mucosal innate immunological system responses.

Keywords (Thai): การติดเชื้อ ภูมิคุ้มกันแบบสืบทอด กลไก เซลล์เยื่อบุมดลูกสุกร ไวรัสพีอาร์อาร์เอส

Keywords (English): Infection, Innate immunity, Mechanism, Porcine Endometrial cell, PRRSV

Research questions

1. Whether the PE cells infected with PRRSV demonstrate the changes of epithelial histology and the PRRSV receptor protein/mRNA expression related to the number of PRRSV-positive cells? What are the different responses to the different genotypes and routes of infection?
2. Whether the PRRSV induce or change the mucosal innate immunological system responses, TLRs expression and related cytokine release and synthesis in the PE cells? What are the different responses to the different genotypes and routes of infection?
3. Whether the PRRSV-re-infection demonstrates the different responses in terms of changes of epithelial histology, related cytokine release and

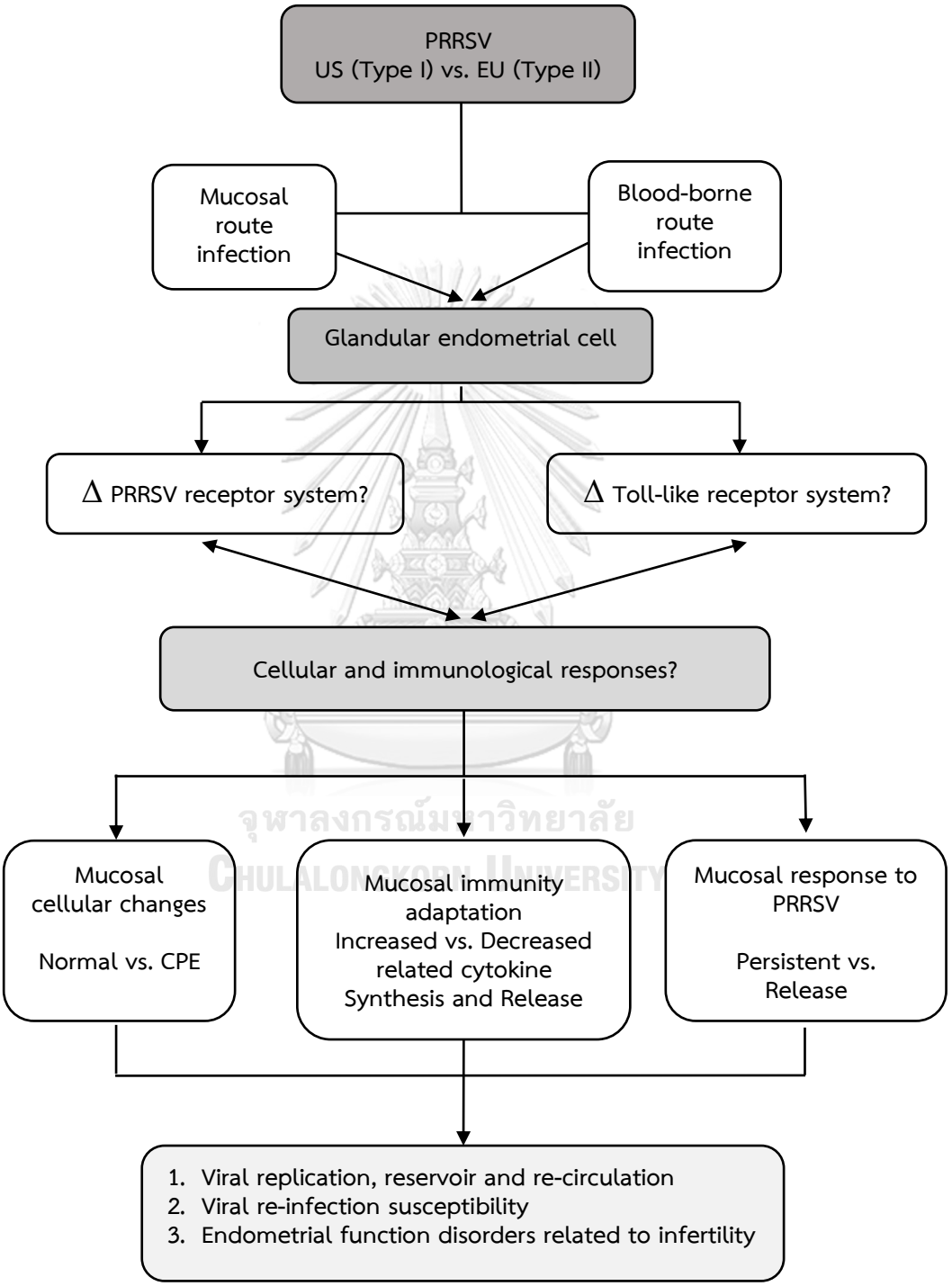
synthesis and the number of PRRSV-positive cells from PE cells primary infected with PRRSV? What are the different responses to the different genotypes and routes of infection?

Hypothesis

The hypotheses of the study are

1. PRRSV can infect and replicate in PE cells through the expression of PRRSV receptors; CD151, CD163, Sn, integrin or vimentin.
2. PE cells infected with PRRSV demonstrate the cellular changes of microscopic characteristics or cytopathic effects (CPE), the expression of PRRSV receptors and TLRs mRNA/protein expressions.
3. PRRSV infection induces immunological responses, the synthesis and release of related cytokines, CCL-2, IL-1β, IL-6, IL-8, IL-10, IFN-γ and TNF-α, leading to the reduction of PRRSV-positive cells.
4. Re-infection with PRRSV demonstrates the cellular (2) and immunological response (3) is different from the earlier PRRSV infection.
5. Different genotypes and routes of PRRSV infection demonstrates the different responses.

Conceptual framework



CHAPTER II
REVIEW LITERATURE

ENDOMETRIUM

Physiology of endometrium

The uterus and uterine horns is an important organ in female reproductive system, which is the site of many physiological processes. The wall of uterus consists of 3 layers; the mucosal (endometrium), the muscular layer (myometrium) and the serosal layer (perimetrium) (Lorenzen et al., 2015). The epithelial cells in endometrium can be categorized into 2 types: the luminal (LE) and the glandular epithelium (GE). At birth, the pig uterus contains only a simple columnar LE but GE are absent. Development of GE occurs after birth and the porcine uterus undergoes maturation by day 120 from birth (Okrasa et al., 2014). Progressive invagination of LE to the stroma begins to develop GE. The GE then forms endometrial gland and plays a role in secretory function, conceptus survival and implantation, and stromal cell decidualization (Filant and Spencer, 2014). During pregnancy, gene expression profiles of the endometrium are dynamic changes distinguishable through the different stages. These changes indicate the diverse patterns that may play a critical role in implantation, endometrial remodeling and fetal development (Kim et al., 2015).

Porcine endometrium during pregnancy

Establishment of pregnancy, consisting of implantation, placentation and maintenance of pregnancy required for full-term fetal development is regulated by conceptus and maternal communication (Geisert et al., 2014). During pregnancy, the glandular endometrium of uterus expresses high secretory activities to produce many enriched substances by which are subsequently secretes to lumen containing; enzyme, growth factors, transport proteins, chemokine, cytokine and prostaglandins (Okrasa et al., 2014). However, the processes involving pregnancy are different among different species. In pig, gestation period spans 113-115 days. Implantation (day 0 -13

of gestation) is the most critical period for determining successful gestation. Implantation of pig uterus is described as an initial placentation including shedding of zona pellucida by embryo, pre-contact with the luminal endometrium, apposition and adhesion. In pig, placentation is a term that describes the formation of the epitheliochorial placenta by interdigitating of luminal endometrium and trophectoderm of embryo (day 13-16 of gestation), followed by complete placental formation (day 30 of gestation) (Geisert et al., 2014).

As other species, implantation in pig requires inflammatory process mediated by cytokines produced by endometrium and conceptus such as IFN γ and δ, IL-1β, IL-6, leukemia inhibitory factor (LIF) and TNF-α (Okrasa et al., 2014; Waclawik et al., 2017). Besides, Interleukin-1β, IL-6, LIF and TNF also participate in conceptus development and regulation of steroidogenesis (Okrasa et al., 2014). During maternal recognition, estrogen from conceptus switches endometrial production and secretion of prostaglandin to luteothophic PGE2 (Waclawik et al., 2009). Meanwhile, the luteolytic effect of PGF-2α is abolished by shifting the circulation of PGF-2α from utero-ovarian vein to the uterine lumen (Bazer and Thatcher, 1977). PGE2 secreted by conceptus and endometrium also plays a role in positive regulation of PGE2 production by endometrium (Waclawik et al., 2009). The secretory activity of endometrium and participating in maintaining progesterone production from maternal corpus luteum is important for establishing and maintaining pregnancy. Increasing uterine secretory activities during pregnancy and modulating maternal immune response is also critical for accomplishing normal gestation.

Regulation of endometrium function

Principally, endometrial functions are under the regulation of ovarian steroid hormones, including E_2 and progesterone (P_4). E_2 promotes proliferation mainly through a proliferative phase and primes the P_4 receptors before secretory phase. The principle effect of P_4 is suppression of E_2 proliferative effect, regulation of endometrial secretion and maintenance of pregnancy (Clancy, 2009).

In addition, the endometrium is regulated by cytokine, which is in the autocrine, paracrine and endocrine manners, during many processes in particular establishment and maintenance of pregnancy (Waclawik, 2011; Geisert et al., 2012; Prins et al., 2012; Geisert et al., 2014; Salleh and Giribabu, 2014). Endometrium itself also plays a role in an innate immune response by secreting many kinds of pro-inflammatory cytokines (IL-1 and IL-8), anti-inflammatory cytokine (IL-6) and IFNs. However, the induction of innate immunity of porcine can respond to both maternal-embryo interaction, uterine remodeling and against pathogen invasion (Waclawik, 2011; Geisert et al., 2012; Prins et al., 2012; Geisert et al., 2014; Salleh and Giribabu, 2014). Therefore, innate immunity in PE during the pregnancy to trigger the inflammation cascade should be tightly regulated in order to prevent rejection of the semi-allogeneic conceptus. The exaggerated activation of inflammation in response to pathogens may be harmful to conceptus survival.

Infectious causes of embryonic fetal death

Indeed, the failure of reproduction in animals can be directly affected by a wide range of infectious pathogens or indirectly by the placentitis. However, the presence of abnormalities depends on the onset of disease, whether they occur during embryo or fetal development (Pozzi and Alborali, 2012). After fertilization, the conceptus is defined as embryo until becoming the completed organogenesis. Thereafter, the fetus is called when the conceptus has complete organogenesis until reaching the last trimester of gestation (Givens and Marley, 2008). The occurrence of the disease during different reproductive stage may demonstrate the different clinical outcomes. The sow may irregularly return to estrus if embryos are affected during or within 2 weeks after insemination. At 3^{rd}- 4^{th} weeks of pregnancy, affecting embryos can be found as the expelled small vesicle on the floor. If the embryos are affected after 2 months of gestation, stillbirths and mummification may be presented (Pozzi and Alborali, 2012).

Mostly, the embryonic death occurring to all embryos in utero, which is usually found during implantation through the development of fetal placenta, was caused

by non-infectious factors, i.e. the imbalance of hormone system and receptors. On the other hand, fetal death mummification, and still birth usually occur depending on the time of fetal insult during gestation. Extensively maternal illness, i.e. viral infection is usually associated with loss of pregnancy control and epidemic of fetal death. The causes of death are often caused by infectious agents that directly effects on the fetus and/or placenta, or indirectly through mother (Christianson, 1992).

Table 1 Gestational period; embryo development and main clinical findings reviewed by Pozzi and Alborali (2012)

Day from AI Stage of development	0-14 Morula	14-30 Before calcification	30-70 Bone calcification	>67 Immuno-competence	105-115	farrowing
Infectious induced	Embryo death	Embryo-death, absorption, expulsion, early abortion	Mummification or reabsorption, abortion	Fetal-death, mummification or maceration	Late abortion, early farrowing	Stillbirth, prepartum or intra-partum death
Clinical findings	None	May find small vesicles	May find small vesicles	abortion	Abortion, early farrowing	Stillbirth, agalectesia lungs
	RIE In cycle	RIE In cycle and not	May retain mummies until farrowing	Mummies maybe presented		

AI: Artificial insemination, RIE: return to estrus

Several pathogens causing reproductive failure in pigs have been reported. They affect the different reproductive stage. Additionally, the different outcomes in different fetuses may be present due to the physiology of presenting multiple fetuses in utero. Predominantly viruses i.e., PRRSV; porcine parvovirus; porcine circovirus type 2; and porcine pseudorabies virus are associated as the porcine reproductive pathogens (Givens and Marley, 2008). However, the clinical sign of sows affected by PRRSV but other pathogen results, and transplacental transmission of PRRSV are most common in the late trimester of gestation leading to stillbirths,

autolytic or mummified fetus and the combination of normal or weak PRRSV-carrier piglets.

INNATE IMMUNITY SYSTEM

Innate immune mechanisms exist in all organisms from the microorganism to the mammal species. The immediate responses of innate immunity provide the front line of protection against pathogen invasion prior to activating adaptive immune response. Appropriate innate response is significant for the most viral diseases, which consider whether the infection being eradicated or persisted (Beutler, 2004; Pancer and Cooper, 2006). There are several kind of cells, including macrophages, monocytes, dendritic cells, natural killer cells, mast cells, eosinophils and neutrophils involved in an innate immunity (Stanley and Lacy, 2010). However, all nucleated cells, including epithelium, are capable to provide innate immune responses when exposed to the viral infection (Beutler, 2004).

Toll-like receptors

To activate the innate immune response, the microorganism disclosing the specific molecules motifs conserved within a class of microbes, which is so called pathogen-associated molecular pattern (PAMPs). Basically, PAMPs are recognized by the sensor of immune cells. Besides immune cells, many proteins of host cells, can detect molecules typical for the pathogens, and has been classified as pattern recognition receptors (PRRs). Toll-like receptors (TLRs), an evolutionary conserved PRRs, have a crucial role in recognizing several PAMPs.

TLRs are expressed on various immune and non-immune cells, including macrophages, lymphocytes fibroblasts and epithelial cells (Akira, 2006). In mammals, TLRs 1-10 have been discovered. Each type of TLR recognizes distinct PAMPs ligands and activates the distinct pathway (Table 2). Moreover, host cell death and tissue injury resulting from inflammatory responses and the release of host cellular components to the extracellular environment can be a ligand for TLRs. They are known as “damage-associated molecular patterns” (DAMPs) consisting of lipids,

sugars, metabolites, and nucleic acids. Although binding of DAMPs to TLRs leads to elimination of pathogens. This might activate the chronic inflammation or develop the autoimmune disease. Therefore, dysregulation of TLRs by sensing both PAMPs and DAMPs can develop pathology to their host (Jounai et al., 2012).

Six of TLRs are involved the responses to viral infection. The cytoplasmic TLRs, TLR2 and TLR4, recognize the viral proteins, whereas the intracellular TLRs, TLR3, TLR7, TLR8 and TLR9, detect the viral nucleic acids, either DNA or RNA virus (Akira, 2006).

TLRs signaling pathway (Fig. 1) mediating cellular mechanism respond to PAMPs has been classified into 2 major pathway; (1) MyD88-dependent pathway mediates all TLRs, excepting TLR3, uses to activate mitogen-activated protein kinase (MAPK) pathways and common transcriptional factor NF-κB which resulting induction of pro-inflammatory cytokines; (2) MyD88-independent pathway have been indicated for TLR3 signaling mechanism by using TIR-domain containing adaptor-inducing IFN-β (TRIF) to stimulate IFN regulatory factor 3 (IRF-3) and the expression of IFN which is essential for anti-viral functions. TLR4 signaling is quite unique that can activate both MyD88 and TRIF dependent pathway (Fig. 1). TLR7, TLR8 and TLR9 activate antiviral cytokines, type I IFN production through MyD88. (Dowling and Mansell, 2016). Various components of the virus that recognized by TLRs activate the production of pro-inflammatory cytokines, including IL-1β, IL-6, IL-12 and TNF-α, as well as induction of type I IFNs. However, TLR2 and TLR4 can recognize structural proteins of viral-envelope and results in the production of pro-inflammatory cytokines instead of type I IFNs. Thus, the response to virus by this pathway leads to the inflammation rather than specific antiviral responses.

Particularly, viral nucleic acids are sensed by TLR3, TLR7, TLR8 and TLR9. Their signaling pathway commonly induces both pro-inflammatory cytokines and type I IFNs production (Akira, 2006). Therefore, several genes such as cytokines, a variety of chemokines, production of reactive oxygen species, induction of apoptosis and phagocytosis are regulated by engagement of TLRs. The production of pro-inflammatory cytokines not only promote local inflammation, but also serve as communicating signal between other innate immune cells. Finally, it can link to the

activation of the adaptive immune system (Stanley and Lacy, 2010). Failure to stimulate the appropriated innate immune system may cause the overstated and inadequate response.

Table 2 Toll-like receptors and their ligands reviewed by Takeda and Akira (2004)

Receptors	Ligands	Sources
TLR1	Triacyl lopopeptides Soluble factors	Bacteria and mycobacteria *Neisseria meningitidis*
TLR2	Lipoprotein/lipopeptides Peptidoglycan Lipoteichoic acid Lipoarabinomannan Phenol-soluble modulin Glycoinositolphospholipids Glycolipids Porins Atypical lipopolysaccharide Atypical lipopolysaccharide Zymosan Heat-shock protein 70	Various pathogens Gram-positive bacteria Gram-positive bacteria Mycobacteria *Staphylococcus epidermidis* *Trypanozoma cruzi* *Treponema maltophilum* *Neisseria* *Leptospira interrogans* *Porphyromonas gingivalis* Fungi Host
TLR3	Double-stranded RNA	Viruses
TLR4	Lipopolysaccharide Taxol Fusion protein Envelope protein Heat-shock protein 60 Heat-shock protein 70 Type III repeat extra domain A of fibronectin Oligosaccharide fragments of heparan sulfate Fibrinogen	Gram-negative bacteria Plants Respiratory syncytial virus *Chlamydia pneumoniae 1* Host Host Host Host Host
TLR5	Flaggellin	Bacteria
TLR6	Diacyl lipopeptides Lipoteichoic acid Zymosan	Mycoplasma Gram-negative bacteria Fungi
TLR7	Imidazoquinolone Loxoribibe Bropirimine Single-stranded RNA	Synthetic compounds Synthetic compounds Synthetic compounds Viruses
TLR8	Imidazoquinolone Single-stranded RNA	Synthetic compound Viruses
TLR9	CpG-containing DNA	Bacteria and viruses
TLR10	Not Determine	Not determine

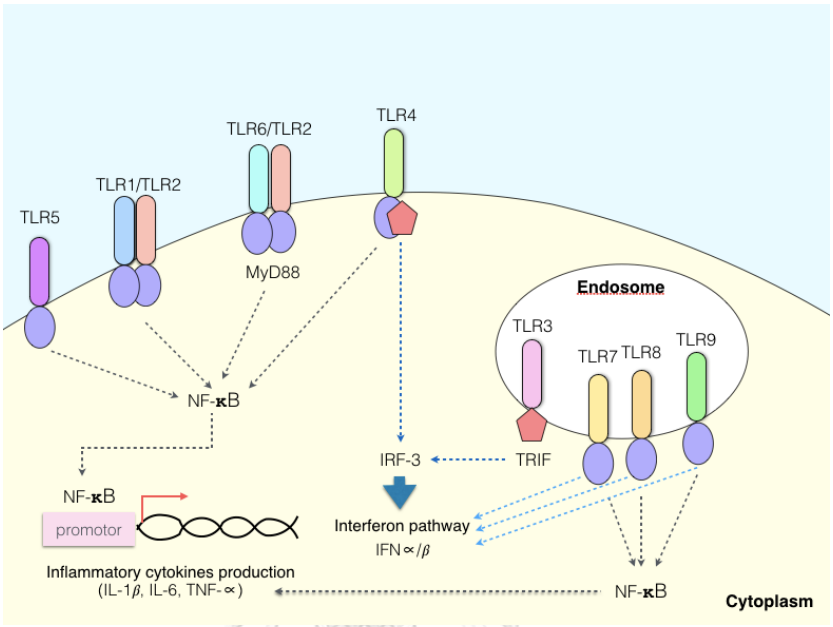


Figure 1 The schematic summarize the classification of toll-like receptors (TLR1-9) and their signaling reviewed by Dowling and Mansell (2016). TLRs signaling pathway are (1) MyD88-dependent pathway mediates all TLRs, excepting TLR3, (2) MyD88-independent pathway have been indicated for TLR3 signaling mechanism by using TIR-domain containing adaptor-inducing IFN-β (TRIF) to stimulate IFN regulatory factor 3 (IRF-3) and the expression of IFN.

Toll-like receptors in female reproduction

There are several kinds of pathogens or non-pathogens interacting to female reproductive tissue, including microorganism, sperm or semi-allogenic fetus, and can be detected by TLRs expressed throughout the female reproductive tract. Apart from infection, TLRs have diverse roles in reproductive tissues implicating in ovulation, fertilization, gestation and parturition (Kannaki et al., 2011). In human endometrial cell line, TLR3 function can be modulated by cellular treatment with E_2. Although other TLRs were not observed. Possibly, innate immune response in female

reproductive tissue is fluctuated through the modulation of TLRs function, indicating the regulatory role of ovarian sex steroids on the TLR expression (Lesmeister et al., 2005). During secretory phase of endometrium, TLRs are expressed higher than those in proliferative phase. In general, the immunological phases in normal pregnancy, consisting of 1) pro-inflammatory environment during early embryo implantation; 2) Immunotolerance at mid-pregnancy and 3) pro-inflammatory environment at the last stage of pregnancy prior to laboring requires the proper function of TLRs system.

In human, TLR4 regulates cytokine production by trophoblast at first stage of pregnancy and prevent gram-negative bacteria infection at decidua cells of human uterus. Preterm labor of human is the consequence of TLR2 activation leading to apoptosis of placenta. Moreover, expression of TLR2 are up-regulated during labor, indicating that activation of TLRs may be important for the term of laboring (Amirchaghmaghi et al., 2013).

Several TLRs expression can be observed in domestic animal, including cattle, sheep, dog, cat, pig and chicken. Beside infection, the role of TLRs in tissue remodeling has been suggested. Persistent up-regulating of TLRs expression at cumulus oocyte complex regulates the pro-inflammatory process during ovulation by releasing pro-inflammatory cytokine to promote the follicle rupture and release of oocyte (Kannaki et al., 2011).

However, pathogenesis and disorder of reproductive tract are also involved with TLRs activation. Up-regulation of TLR4 by maternal infection and maternal obesity result in placentitis and abortion in sheep. In sow, mastitis correlates to TLR2 up-regulation (Kannaki et al., 2011). In addition, the diseases linked to the inflammation, such as pyometra in dogs, revealed the up-regulation of TLR2 and TLR4 suggesting that some factors response to microorganism can modulate the TLRs expression (Chotimanukul and Sirivaidyapong, 2011; Chotimanukul and Sirivaidyapong, 2012). Understanding the dynamics of TRLs during host-viral interaction may be beneficial for prevention of viral infection. However, little is known about TLRs functions respond to PRRSV in the reproductive organs of pregnant sows and gilts.

Genital innate immune system

The genital immune system is a part of the mucosal immune system, which is generally composed of innate (non-adaptive; non-specific) and adaptive (specific) immune systems. In pigs, physiological infiltrations of immune cells in the endometrium are vary depending on reproductive cycle, sow status and insemination. The largest population of leukocytes found in the endometrium of sows is lymphocytes. $CD8^+$ (Cytotoxic T lymphocytes) are the most common found than $CD4^+$ (Helper T lymphocytes) in luminal epithelium. By contrast, $CD4^+$ is higher expression in sub-epithelial connective tissue than $CD8^+$. Neutrophils play a role in the first line of defense, and it is physiologically up-regulated during proestrus and estrus to eliminate the pathogen and foreign material during mating/insemination, leading to sterile condition at the time of pregnancy (Dalin et al., 2004).

Apart from leukocytes, endometrial cell itself acts as innate immune cells by building a physical barrier and secreting many kinds of cytokines against pathogen invasion (Lorenzen et al., 2015). In addition, tight junction provides the strong physical barrier of epithelial cells. The loosen of tight junction reflected by the decrease of transepithelial resistance allows the microorganism across the epithelium to blood circulation. Since the proliferative lesions of cutaneous microvasculature associated with the increased vascular permeability were reported in virulent genotype PRRSV infection (Scruggs and Sorden, 2001), other epithelia including the endometrial epithelia may be the target. The increased permeability of the glandular endometrium by PRRSV infection may affect the secretory function of mucosa leading to reproductive organ disorder. Besides the function of physical barrier, endometrial cells can secrete several factors, which acts as a chemical barrier.

Many cytokines, chemokines and growth factors are also secreted by endometrial cells to neutralize pathogens. In addition, they regulate the various events of endometrial cells. Many factors are involved embryo implantation stage and support embryo implantation, including IFN-γ, IL-1β, IL-6 and TNF-α, CCL-3, CCL-4, CCL-5, FGF2, granulocyte colony-stimulating factor (G-CSF), IL-1β, IL-6, IL-8, leukemia inhibitory factor (LIF), platelet-derived growth factor (PDGF) β, TNF-α and

vascular endothelial growth factor (VEGF) by promoting inflammation during blastocyst implantation (Srivastava et al., 2013).

In response to bacterial lipopeptides, LPS, and IL-1β, endometrial cells secrete IL-8 and IL-6 (Cronin et al., 2012; Turner et al., 2014; Healy et al., 2015). The anti-viral response of uterine epithelial cells has been reported by secreting the pro-inflammatory cytokines TNF-α, IL-6, GM-CSF and G-CSF, as well as the chemokines CXCL-8/IL-8, CCL-2/MCP-1 and CCL-4/MIP-1 (Schaefer et al., 2005). They initiate the inflammatory response and recruit immune cells to the site of infection to clear the pathogens. However, the local innate immune system of endometrium responded to PRRSV has never been reported. Imbalance of host-pathogens interaction producing overstated or inadequate innate immune response may associate with pathogenesis or persistence of pathogens.

PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME (PRRS)

Structural biology of PRRSV

PRRSV has been identified as an enveloped, positive-strand RNA virus in the family of Ateriviridae, order Nidovirales along with equine arteritis virus (EAV), lactate dehydrogenase-elevating virus (LDV) of mice and simian hemorrhagic fever virus (SHFV) (Benfield et al., 1992; Cavanagh, 1997; Snijder and Meulenberg, 1998). The genome of PRRSV, single stranded RNA, is enclosed by nucleocapsid protein (N). The major envelope viral protein, GP5 and M, form a heterodimer structure. The minor structural proteins are GP2a, E, GP3 and GP4 (encoded from ORFs2-4), which forms multimeric complex (Fig. 2).

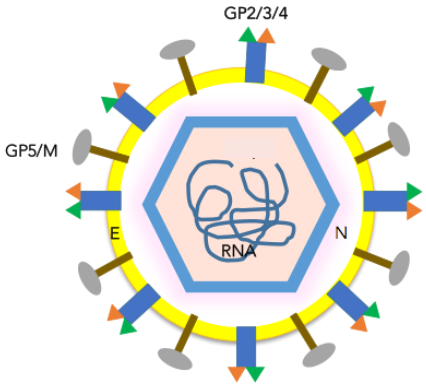


Figure 2 Schematic representation of the structure of PRRSV particle reviewed by Music and Gagnon (2010). PRRSV nucleic acid (ssRNA) is in the core and surrounded by nucleocapsid protein (N). The viral envelop protein, is so called PRRSV structural proteins consist of M/GP5 heterodimer and GP2/GP3/GP4/E multimers.

A 15Kb RNA of PRRSV genome contains at least 9 open-reading frames (ORFs) (Fig. 3) (Dea et al., 2000). Like other Nidovirales viruses, ORF1 of PRRSV, which locates at 5' terminal of PRRSV genome, consists of ORF1a and ORF1b. The ORF1a and ORF1b encode polyproteins and pp1ab, respectively. The polyproteins then are subsequently processed into at least 12 nonstructural (nsp) proteins (Snijder and Meulenberg, 1998). The nsp1 to nsp8 are cleaved from pp1a, while nsp9 to nsp12 are processed from pp1ab. Due to the proteolytic activity, the product from pp1a are responsible for cleavage and processing the other nsp products, whereas nsp9 to nsp12 participate in PRRSV transcription and replication (Snijder and Meulenberg, 1998). The minor glycosylated enveloped proteins (GP2a, GP3 and GP4) are encoded by ORF2, ORF3 and ORF4 and form multimeric complex by the linking of disulfide bond (Wissink et al., 2005). The major glycoprotein GP5 forms a heterodimer with the other major non-glycosylated protein M encoded by ORF5 and ORF6 respectively. The ORF7 encodes the nucleocapsid N protein, which is assembly to the viral genome (Dea et al., 2000).

PRRS virus (PRRSV) has been categorized into 2 distinct genotypes, European (EU) genotype or type I PRRSV, and North America (US) genotype or type II PRRSV (Nelsen et al., 1999), which share only 55-80% genetic identity between genotypes. Many reports demonstrated that there are genetic variety within PRRSV genotypes. Therefore, PRRSV is classified into a several phylogenic cluster within each genotype (Music and Gagnon, 2010).

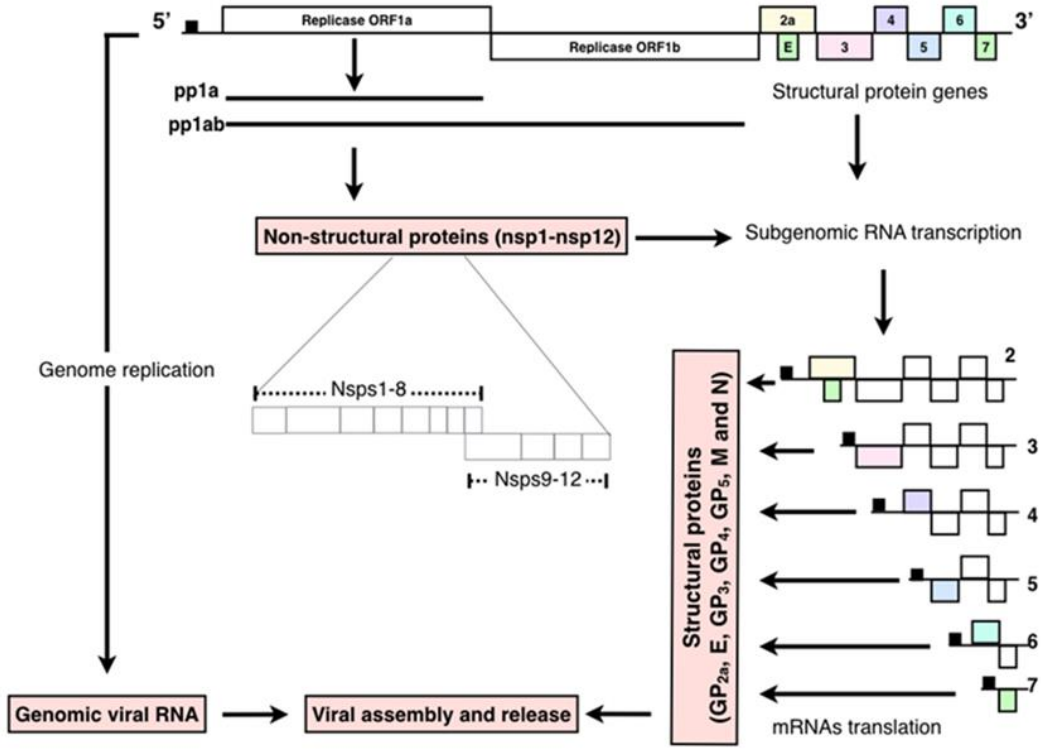


Figure 3 Genome organization and replication of PRRSV. The polyprotein pp1a and pp1ab are expressed from replicase ORF1a and ORF1b. The structural proteins (GP2a, E, GP3, GP4, GP5, M and N) are encoded from subgenomic RNAs 2-7. The pp1a are cleaved at 8 sites to form nsp1 to nsp8, thereby pp1ab are produced nsp9 to nsp12. reviewed by (Music and Gagnon, 2010).

The cell biology of PRRSV entry and infection mediated by the interaction between host cell mediators and PRRSV proteins

Virus is an obligate intracellular pathogen that means the virus is unable to replicate outside the host cells. Viral infection starts when the viral particle contacts the surface of the host cells initiating the complex series of events, including distribution through the host cell membrane surface, binding to receptor, propagating the signal, internalization and releasing the viral genome for replicating their progeny (Maginnis, 2018). Interaction with the cellular receptors is a key regulatory step to initiate the viral infectious life cycle and to define whether the tissue has tropism to the virus. Generally, viruses utilize multiple cellular receptor for infection.

First, viruses commonly bind with low affinity to non-specific receptors expressed by host cell membrane. Secondly, they have interaction with a secondary or tertiary receptors with higher affinity binding in order to be internalized (Maginnis, 2018). The use of multiple receptors is an advantage for virus by increasing binding avidity and allows tightly coordinating during virus fusion or penetration (Grove and Marsh, 2011).

Indeed, PRRSV has a very specific cell tropism. It mainly infects cells in macrophage/monocyte lineages, in particular PAMs and other tissue macrophages (Van Gorp et al., 2008; Music and Gagnon, 2010). Dendritic cells are also the main target of PRRSV. However, not all type of dendritic cells has the susceptibility to PRRSV infection. Induction of PRRSV infection to monocyte-derived dendritic cells (MoDCs) showed the susceptible to PRRSV whereas those of primary lung dendritic cells (L-DCs) were not permissive to the virus (Loving et al., 2007). This might be due to the different characteristics that occur during the differentiation pathway of dendritic cell and affect the susceptible to PRRSV (Loving et al., 2007). Like other viruses, the presence of specific receptor of PRRSV in the target cell is the main factor that determines the cell tropism. Many molecules have been currently reported as PRRSV receptors including heparan sulfates, CD163, Sn, integrin and vimentin (Van Gorp et al., 2008).

Heparan sulfate on macrophage had been firstly defined as one of PRRSV receptors. However, solely interaction with heparan sulfate molecules on host cell was incapable to produce infection. In addition, the presence of this molecule is not specific to macrophage. Thus, heparan sulfate are suggested to function only as primary attachment factor (Delputte et al., 2005).

To find other PRRSV receptors on macrophages, alveolar macrophage-specific monoclonal antibodies (mAbs) were generated. It was shown that mAb 41D3, which was identified to mAbs against porcine Sn molecule, was ability to block PRRSV infection (Duan et al., 1998a; Duan et al., 1998b). Expression of recombinant Sn in non-permissive cell lines established PRRSV attachment and internalization through receptor-mediated endocytosis but not replication. It indicates that Sn is important for PRRSV attachment and entry receptor (Vanderheijden et al., 2003).

CD163, type I glycoprotein specific expressed on macrophage and monocyte, was identified as an significant PRRSV receptor that confer PRRSV susceptible by screening the cDNA library of porcine alveolar macrophage comparing to otherwise non-permissive cells (Calvert et al., 2007). The presence of CD163 in MARC-145 non-permissive cell lines allowed productive of PRRSV infection and this infection could be inhibited by CD163-specific antibodies (Calvert et al., 2007). Co-expression of PRRSV minor glycoproteins (GP2 and GP4) with CD163 was observed in PRRSV infected non-permissive BHK-21 cells by immunoprecipitation using CD163 specific antibodies, suggesting that this interaction may play role in viral genome release (Das et al., 2010).

Despite, more cellular molecules are expected to involve the infectious process of PRRSV, for instances, CD151 and vimentin. Other major cellular receptors utilized by viruses can be categorized into cellular adhesion molecules family receptors (CAMS) (Maginnis, 2018). In particular, integrin is an integral membrane protein served as CAMS family and function as including cell-to-cell and cell-to-matrix adhesion, cellular development, cell signaling and repairing process (Farahani et al., 2014). Also, it can be used as viral receptor for viral entry and activation of signaling pathway by a wide range of viruses including PRRSV (Maginnis, 2018). The

mechanism of PRRSV infection by utilizing these molecules has not been established at the present (Van Breedam et al., 2010a).

In summary, as shown in figure 4, Sn and CD163 are the classical PRRSV receptor and required for PRRSV infection. Sn mediates the binding and internalization, whereas CD163 is required for uncoating (Van Gorp et al., 2008). Initially, heterodimer protein of PRRSV, GP5/M, binds to Sn and subsequently internalizes by endocytosis (Van Breedam et al., 2010b). PRRSV genome is released by interaction of GP2/GP3/GP4 to CD163 (Das et al., 2010). After internalization and uncoating, PRRSV replication occur in host cytoplasm like other viruses. Finally, viral RNA and protein are assembly to form the new viral particles (Benfield et al., 1992). It is interesting that molecules of PRRSV might interact to host molecules during infection and causes various possible consequences such as induction of cellular signaling during binding to PRRSV receptor and/or modulation of host immune responses.

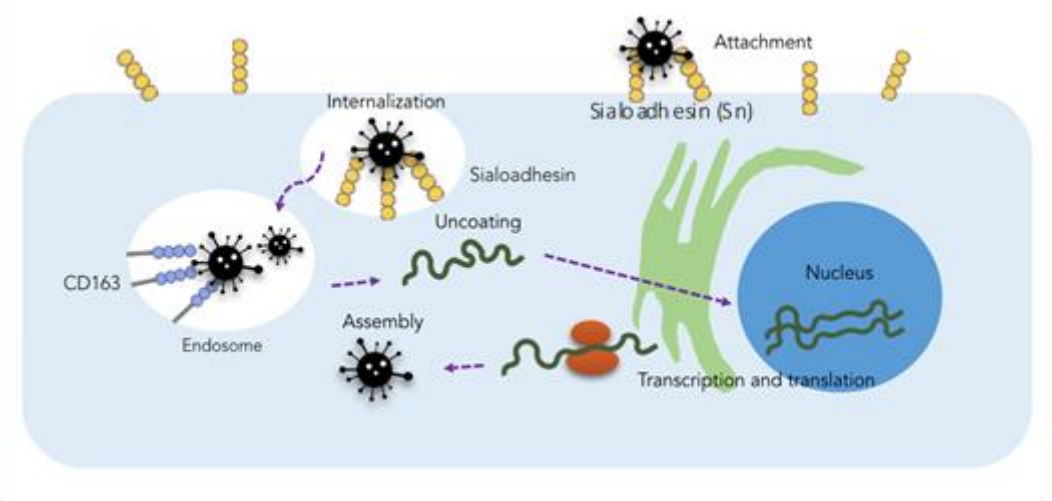


Figure 4 Schematic representation of the cellular entry, uncoating and replication of PRRSV at the PRRSV cell tropism are mediated by CD163 and Sn PRRSV receptor proteins reviewed by Van Gorp et al. (2008).

Pathogenesis of PRRSV

Infection with different PRRSV genotypes leads the different severity of clinical outcomes. PRRSV type II has been reported that caused more severe respiratory distress than the other and resulted in subsequent transplacental infection (Nielsen et al., 2002). By contrast, PRRSV type I has less severity in clinical outcomes (Scortti et al., 2006). Transmission of PRRSV was occurred between pigs by shedding of the virus (horizontal transmission) and transplacental viral shedding (vertical transmission) (Christianson and Joo, 1994). PRRSV infection in sows during pregnancy usually results in abortion and weak-born piglets.

The survival piglets are susceptible to secondary infection and act as a PRRSV reservoir in the herd. As a result, recirculation of PRRSV in the herd is hardly to eradicate. Although there are many commercial vaccines available, the efficacy of those vaccines is still uncertain. The unsuccessful of vaccine is results from the lack of cross protection across the PRRSV genotypes. The inactivated PPRSV vaccines have been suggested, but it was dissatisfied to prevent conceptus infection even the use of homology genotype (Scortti et al., 2006; Karniychuk et al., 2012). The attenuated PRRSV vaccination for gilts is preferred, since it can reduce the number of PRRSV-positive fetuses by lowering pathology and virus replication in the fetal placenta. Unfortunately, attenuated vaccine virus turns to virulence causing the fetal death or transplacental spread of the attenuated vaccine virus from mother to fetuses has also been reported (Scortti et al., 2006; Karniychuk et al., 2012).

In respiratory infection, it is clear that PRRSV primary infects PAMs as the natural target cells and entry the circulation to transport the viral particles to other tissues (Van Gorp et al., 2008). Nucleocapsid (N) protein, the highly immunogenic protein of PRRSV, localizes in the nucleus and plays a role in cellular pathogenesis (Music and Gagnon, 2010). During PRRSV infection, N protein might initiate host cellular transcription factor and regulate host cell gene expression (Sang et al., 2009). The early immunogenic responses in PRRSV-infected pigs are generated against N protein. The heterodimer of GP_5 and M is also an important molecule for PRRSV infection (Snijder et al., 2003). Not only is the key molecule of viral assembly

(Verheije et al., 2002), but GP5/M heterodimer also confers the specific PRRSV neutralizing antibodies (Lopez and Osorio, 2004; Music and Gagnon, 2010).

In the reproductive model (Fig. 5), some hypotheses of PRRSV transplacental transmission have been postulated 1) PRRSV travelling along the maternal-fetal interface via infected macrophage 2) free PRRSV particle shedding from maternal viremia to fetal placenta and 3) endometrial infection from PRRSV infected macrophage viral shedding. (Karniychuk and Nauwynck, 2013). The author concluded that the possible mechanism is the migration of PRRSV-infected macrophage from maternal circulation to fetal tissue via the Trojan mechanism (Karniychuk and Nauwynck, 2013). The PRRSV-infected fetus showed the macroscopic lesion with the PRRSV-positive but not being the cause of abortion (Karniychuk et al., 2013). However, the PRRSV replication at endometrium and placenta themselves may be an important side for PRRSV transplacental infection because PRRSV replication was detected only at late-term gestation in the endometrium and placenta (Karniychuk et al., 2011). Apoptosis has also been observed in PRRSV-positive cells and surrounding cells at the fetal implantation site (Karniychuk et al., 2011). This indicates that PRRSV-infected cells in the endometrium/placenta can indirectly destroy the surrounding cells by interfering expression of extracellular matrix proteins and secreting cytotoxic substances (Karniychuk et al., 2013).

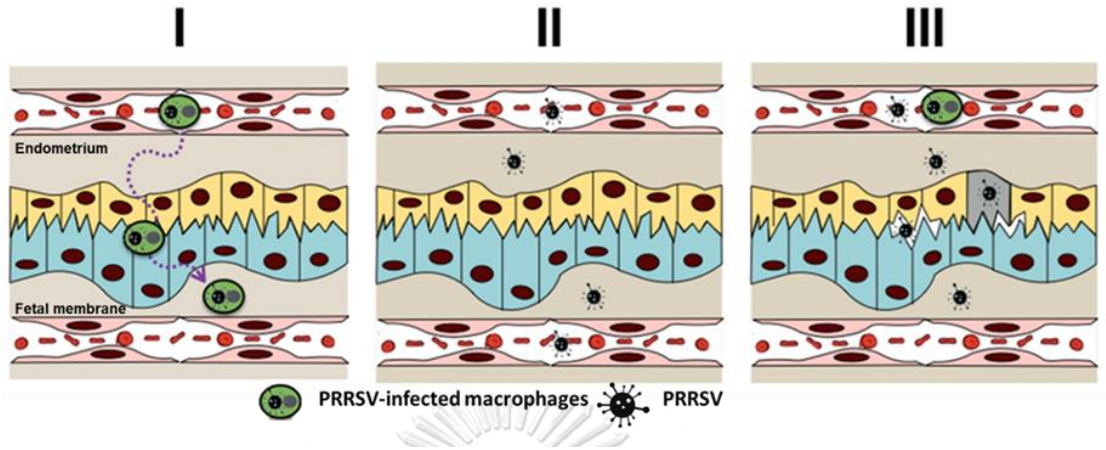


Figure 5 Schematic represents a possible mechanism of PRRSV transplacental transmission reviewed by Karniychuk and Nauwynck (2013) I) PRRSV is carried by the infected macrophages and migrated from the mother to the fetus. II) Free particle of PRRSV is spread through the maternal tissue and fetal placenta. III) PRRSV is transmitted directly from infected endometrial macrophages to the endometrium and subsequently through the fetal tissues.

Interaction between PRRSV and innate immunity

The cause of persistent PRRSV in swine production is the alteration of porcine innate immune in response to PRRSV. This virus compromises the host immune response, including modulating the cytokine production, reducing receptor expression and phagocytosis and well as intervening the recognition of the virus (Sang et al., 2011). The standard model for determination of PRRSV immunopathology is PAMs and some permissive cell lines. Culture PRRSV in PAMs and PBMC collected from infected pigs has been demonstrated that PRRSV can modulate expressions of TLR2, TLR3, TLR4, TLR7, TLR8 and TLR9, which are viral sensing TLRs (Liu et al., 2009; Tu et al., 2015; Zhang and Yoo, 2015). Alterations in TLRs expression in response to PRRSV infection lead to modulation of pro-inflammatory cytokine secretions, especially IL-1β, IL-6, IL-10, TNF-α and IFN-α (Katze et al., 2002; Liu et al., 2009; Borghetti et al., 2011; Tu et al., 2015).

As aforementioned, the major effectors of innate immune responses to virus contain type I IFNs and some anti-microbial peptides. Type I IFNs are prominent in antiviral activities by inactivating viral responses and limiting viral replication. During PRRSV infection, IFN-α is highly potential to inhibit PRRSV by controlling the viral infection in PAMs and MARC-145 cells (Sang et al., 2010; Sang et al., 2011). However, IFN-α is down-regulated in response to PRRSV infection (Sang et al., 2010). Moreover, production of anti-microbial peptides and their activity in the lung of piglets are suppressed by PRRSV (Sang et al., 2009). The up-regulated IL-10 and glucocorticoid production associated with PRRSV infection resulting in immunosuppression has also been reported (Borghetti et al., 2011). In late gestation pigs, PRRSV infection revealed the induction of IFN-α, CCL-2, TNF-α and IFN-γ (Rowland, 2010; Kyuno et al., 2014). Increased Th1 pro-inflammatory cytokines during pregnancy, especially TNF-α and IFN-γ, caused the spontaneous abortion in PRRSV-infected dams (Sykes et al., 2012). The evidences indicate that PRRSV infection might contribute to deactivation host innate immune response.

Therefore, the deficiency of innate immunity in response to PRRSV infection complicates the disease outcomes and increases the host mortality. It is also questioned that whether PRRSV can modify the local innate immune system in endometrium enhancing viral replication and transmission in the endometrium and placenta leading to the disorders of the reproductive organs.

PRIMARY PORCINE ENDOMETRIAL CELL

Primary porcine endometrial cells are the primary cells that generated from glandular epithelia of porcine endometrium. PE cells were characterized as epithelial cells by presenting cytokeratin-18, the intermediate filament expressed in all kinds of epithelial cell (Deachapunya and O'Grady, 1998). **Functional polarity of PE cells** growing in the membranous insert filter was demonstrated consisting of 1) structural polarity 2) apical and basolateral differential protein secretion 3) preference for the secretion of prostaglandins 4) differential ion transportation between apical and basolateral border (Bowen et al., 1996; Deachapunya and O'Grady, 1998).

The structural polarity was determined by the development of complexity of tight junctions, change in cellular morphology and elevation of epithelial resistance. After seeding into an artificial membrane filter, TER of PE cells was low and gradually increased to the maximum value at 3-4 days (Deachapunya and O'Grady, 1998). Forming of junctional complexes at the subapical border produces the differential compartment of epithelial cell into the apical border, the side that exposes to the lumen, and basolateral border, the side that contact with the adjacent cells and underlying connective tissue (Cereijido et al., 1993). Likewise, the presence of tight junctions in PE cells results in separation into apical and basolateral compartment, which similar to the epithelial lining of endometrial layer in uterus.

The preference of protein secretion by PE cells between apical and basolateral compartment is different. Protein secretion into the apical compartment of PE cells composed of higher molecular mass proteins and higher total protein secretion than those of basolateral secretion (Bowen et al., 1996). The mechanism of ion transportation by polarized PE cells were demonstrated that the different ion channels located at the different side of membrane. Apical membrane expressed the Cl^- channel responsible for apical Cl^- secretion. By contrast, basolateral membrane comprised of Cl^- channel, $Na^+/K^+/Cl^-$ cotransport, cAMP-activated K^+ channel and Na^+/K^+ ATPase, which created the electrical gradient and maintain the homeostasis of membrane potential (Deachapunya and O'Grady, 1998).

PE cells have functions similar to native glandular endometrial cells *in vivo*. In PE cells, protein expression of α-estrogen receptor has been reported and was modulated by estrogen and phytoestrogen supplementation (Poonyachoti et al., 2008). Some functions of PE cells were regulated by estrogen and phytoestrogens, genistein and daidzein, through the estrogen receptor. For example, gene expression of tight junction and epithelial resistance of PE cells was improved by genistein and daidzein treatment (Kiatprasert et al., 2015), and the production of antimicrobial peptide in PE cells was modulated by 17β-estradiol, genistein and daidzein (Srisomboon et al., 2017). One critical function of uterus responsible by glandular endometrial cells is secretory activity. PE cells also elicit the secretory function; i.e. ion secretion, prostaglandin, leukotriene and antimicrobial peptide, which are

supervised by hormone, cytokine and inflammatory (Bowen et al., 1996; Deachapunya and O'Grady, 1998; Jana and Czarzasta, 2016; Srisomboon et al., 2017).

Taken together, PE cells provide a variety of reproductive and immunological functions that resemble to native endometrial cells. Therefore, PE cell is a model of choice suitable for evaluating host-pathogen interaction, cell-cell interaction and action of hormone and cytokines.

CHAPTER III
MATERIALS AND METHODS

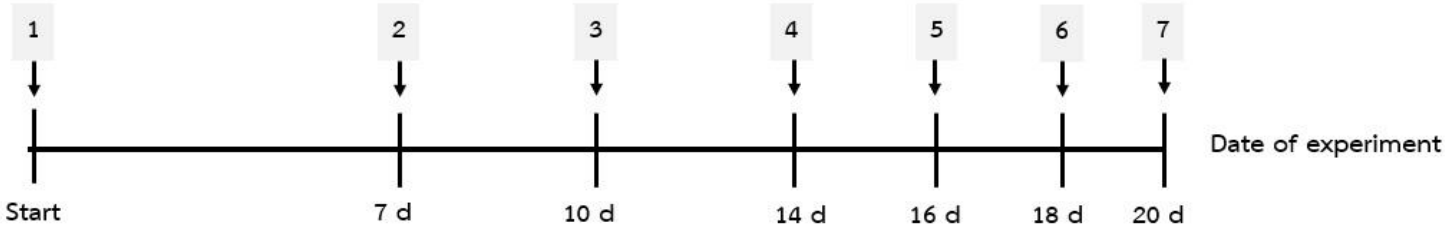
To determine the cellular physiological and immunological responses of PE cells following PRRS re-infection, generation of PE cells, isolation of PRRSV and PRRSV infection were performed initially each part in similar manner.

3.1 Chemical and materials

Chemical for porcine's Ringer solution (NaCl, KCl, $CaCl_2$, $MgCl_2$, $NaHCO_3$, NaH_2PO_4, Na_2HPO_4), cocktail-protease inhibitor and other chemical cellular grade (tween, chloroform, ethanol, isopropanol H_2O_2 and methanol) were purchased from Sigma Chemical Co. Ca^{2+}/Mg^{2+} free phosphate saline buffer for cell culture (PBS), collagenase type I, fetal bovine serum (FBS), Dulbecco's Modified Eagle Medium (DMEM), non-essential amino acids, 0.25% trypsin/EDTA, kanamycin and penicillin-streptomycin were purchased from Gibco (CA, USA). Agarose was purchased from (Seakem, ME, USA),

All cell culture vessels (24 mm microporous membranes in 6-well plate, T25 flask and 100 mm cell culture dish) and 96-well ELISA plates were purchased from Corning (MA, USA).

Primary antibodies for evaluating PRRSV receptors, including goat-anti-CD151, goat-anti-CD163, goat-anti-integrin, goat-anti-integrin and mouse-anti-vimentin, and donkey-anti-goat HRP-conjugated secondary antibody were purchased from Santa Cruz biotechnology (CA, USA). Primary antibody for PRRSV-GP5 protein (rabbit-anti-PRRSV) was purchased from Biorbyt Ltd., (Cambridge, UK) Avidin-biotin-enzyme complex kit consists of HRP-conjugated secondary antibody universal anti-mouse/rabbit IgG, DAB and hematoxylin were purchased from Vector Laboratories (CA, USA).



1 Uterine collection and generation of PE cells

2 PE cells subculture and seeding into T25 flask and 24 mm transwell in 6-well plate

3 **PRRSV infection in T25 flask** at 90% confluent

4 **T25 flask:** Total RNA isolation for real-time RT-PCR at 4 dpi

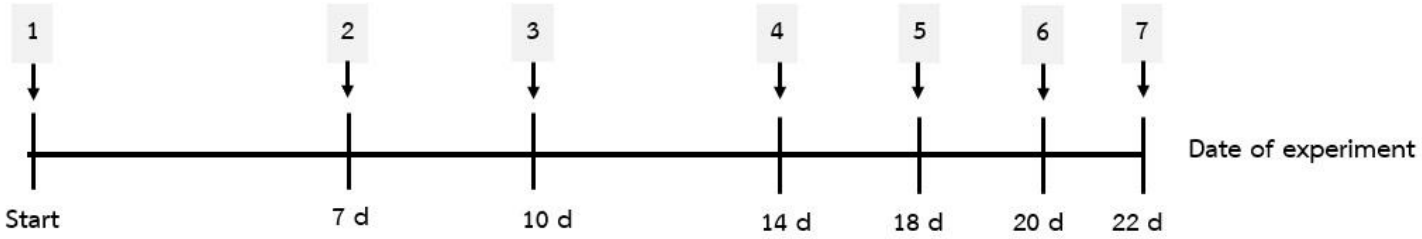
24 mm transwell: Media collection for ELISA at 0 dpi

PRRSV infection at transepithelial resistance = 600-700 Ω

5 6 7 **24 mm transwell:** Media collection for ELISA at 2, 4 and 6 dpi

*** CPE was daily observed and media were refreshed in an alternate day*

Figure 6 Timeline for experiment part I and part II



1 Uterine collection and generation of PE cells

2 PE cells subculture and seeding into T25 flask and 24 mm transwell in 6-well plate

3 1st PRRSV infection in T25 flask at 90% confluent

4 T25 flask: 2nd PRRSV infection at 4 day post infection (dpi)
24 mm transwell: 1st PRRSV infection PRRSV infection at transepithelial resistance 600-700 Ω

5 T25 flask: Total RNA isolation for real-time RT-PCR at 8 dpi
24 mm transwell: Media collection for ELISA at 0 day post-reinfection (= 4 dpi)
Filter collection for IHC at 0 day post-reinfection (= 4dpi)
2nd PRRSV infection PRRSV infection at transepithelial resistance 600-700 Ω

6 7 24 mm transwell: Media collection for ELISA at 2 and 4 days post-reinfection (= 6 and 8 dpi)

*** CPE was daily observed and media were refreshed in an alternate day*

Figure 7 Timeline for experiment III

3.2 Isolation and cultivation of PE cells

Uterine horns of slaughtered finishing gilts (4-6 months old; Thai commercial breed) were obtained from governmental qualifying slaughter house in Bangkok, Thailand. After the removal, tissues were maintained in the ice-cold porcine's Ringer solution containing in mM; 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 during the transportation. According to the protocol of Deachapunya and O'Grady (1998), the tissue was cut into small pieces, washed in Ca^{2+} and Mg^{2+}-free PBS containing 100 µg/ml kanamycin sulfate, 100 U/ml penicillin and 100 mg/ml streptomycin. The muscle layer was then stripped out, and the mucosa was minced and digested 24 hr at 37°C with 0.2% collagenase type I in DMEM. Digested tissue was filtered through a mesh filter (40 µm pore size), and sediment for 15 min at room temperature for 3 times. According to the gravitational sedimentation method, the precipitated pellets containing endometrial glands were isolated from surface endometrium. The isolated endometrial glands were resuspended with growth media, 10% fetal bovine serum (FBS) in DMEM containing 100 U/ml penicillin, 100 µg/ml, streptomycin, 100 µg/ml kanamycin, 1% non-essential amino acids and 10 µg/ml insulin and plated in 100 mm dish for allowing PE cell regeneration from endometrial glands. After incubation in media at 37°C in humidified atmosphere of 5% CO2 in air, PE cells were pipetted to remove the excessive glands and replaced with fresh media. PE cells were plated and maintained in growth media until reaching 90% confluent (~48 hr) prior to performing the experiment. Upon the confluents, PE cells were dissociated from cell culture dish with 0.25% trypsin with 1 mM EDTA (0.25% trypsin/EDTA), and sub-cultured to the appropriate cell culture plate for each experiment. Endometrial tissue and primary cell culture contaminated with Mycoplasma spp., swine fever or PRRSV were excluded when they were positive to the multiplex RT-qPCR detection kit (Microplasma 16s Ribosomal RNA Gene genesis® Standard kit, Primerdesign, Camberley, UK; Virotype® CSFV RT-PCR kit, QIAGEN, Leipzig, Germany; Virotype® PRRSV RT-PCR kit, QIAGEN, Leipzig, Germany).

3.3 Isolation, purification and confirmation of PRRSV

Thai PRRSV field-isolate genotypes I and II were isolated from infected lung of piglets at Farm Animal Hospital, Faculty of Veterinary Science, Chulalongkorn University, Nakorn Pathom, Thailand. The genotypes of PRRSV infection were determined by the certified pathologist and veterinarian Dr. Suphot Wattanaphansak, and multiplex RT-qPCR technique for determining PRRSV genotypes was performed using commercial kit (Accessquick™, Promega). The used primers were included N26: GCCCTAATTGAATAGGTGAC; FT1: AGAAAAAGAAAAGTACAGCTCCGAT; and N26/FT2.1: GTGAGCGGCAATTGTGTCTGTCG that were specific to ORF7 of type I / type II, ORF 7 of type I and ORF 7 of type II, respectively. According to the protocol of Meng and co-workers (Meng et al., 1996), lungs tissues weighting of 2.3 grams were minced and homogenized in cold DMEM 15 ml. The homogenized tissue was then centrifuged at 10,000 *g* at 4ºC for 10 min. Supernatant was then collected and filtered with 0.2 µm filter and used as the inoculum. The PRRSV titers at the concentration of $TCID_{100}$/2ml determined in the standard PRRSV target cells, African green monkey kidney MARC-145 cells followed the protocol as described by Ding and colleagues (Ding et al., 2012). Briefly, MARC-145 cells obtained from ATCC and already available in our laboratory were cultured in 25 cm^2 flask (Costar®, Corning, MA, USA) with maintaining media; 5% FBS DMEM with 100 µg/ml kanamycin sulfate, 100 U/ml penicillin and 100 mg/ml streptomycin and incubated with the filtered viral supernatant. After 1 hr of incubation, the infected MARC-145 was washed and maintained with the fresh media for 2-6 days. Upon the CPE was detected (usually at 4 days post-infection; dpi), cells were fixed in 4% paraformaldehyde for the immunohistochemistry analysis.

3.4 Infection of PRRSV to PE cells

PE cells were plated at the concentration of $1x10^6$ cells ml^{-1} in 24 mm microporous membranes and T25 flask (Costar®, Corning, MA, USA) with maintaining media as aforementioned for 7 days. During the cultivation, the fresh media were replaced every 48 hr. To examine the routing effect of PRRSV transmission, the PE cell monolayers were either apically or basolaterally incubated with solution isolated from mock infection (PRRSV-negative lungs), PRRSV type I-positive lung or PRRSV type

II-positive lung for 1 hr at 5% CO_2, 37ºC. The infection in PE cells was performed duplicately (n= 5 pigs). Meanwhile, a 5 ml the PRRSV viral solution or mock was inoculated to PE cells in 25 cm^2 flask. After 1 hr of adsorption, all PRRSV inoculum solution was washed and replaced with the fresh maintaining media for 2-6 days. During the experiment, cell morphology/CPE of PE cells infected by PRRSV were observed daily under the light microscope digital camera (BX50F and UC50, Olympus, Tokyo, Japan). At 2, 4, 6 dpi, the media were collected from apical and basolateral compartment, and fresh maintaining media were replaced. The infected membranes were collected and fixed in 4% paraformaldehyde at 2, 4 and 6 dpi for the immunohistochemistry assessment. One membrane of each group represented 1 pig (n=5 pigs). Each membrane containing monolayer of PE cell was cut into 0.5 cm^2 and was randomly selected to evaluate occurrence of CPE, PRRSV-GP5 positive and cellular expression of PRRSV mediator proteins. The media collected from infected PE were preceded to observe the release of PRRSV by infecting to MARC-145 followed the same protocol as described in 3.3.

3.5 Determination of PRRSV infection in PE cells by qRT-PCR

To determine the expression of PRRSV nucleic acid, multiplex PRRSV RT-qPCR was performed using commercial kits (Virotype®, Qiagen, Hilden, Germany). Tissue homogenate containing PRRSV from viral isolation was performed RT-qPCR to confirm the expression of PRRSV. PE cells were observed the expression of PRRSV nucleic acid prior to performing experiments to avoid the tissue pre-contaminated with PRRSV and following the infection step to confirm the presence of PRRSV. The protocol of PRRSV RT-qPCR followed the instruction of the kit. The positive sample will be considered at the threshold cycle (C_t) <35.

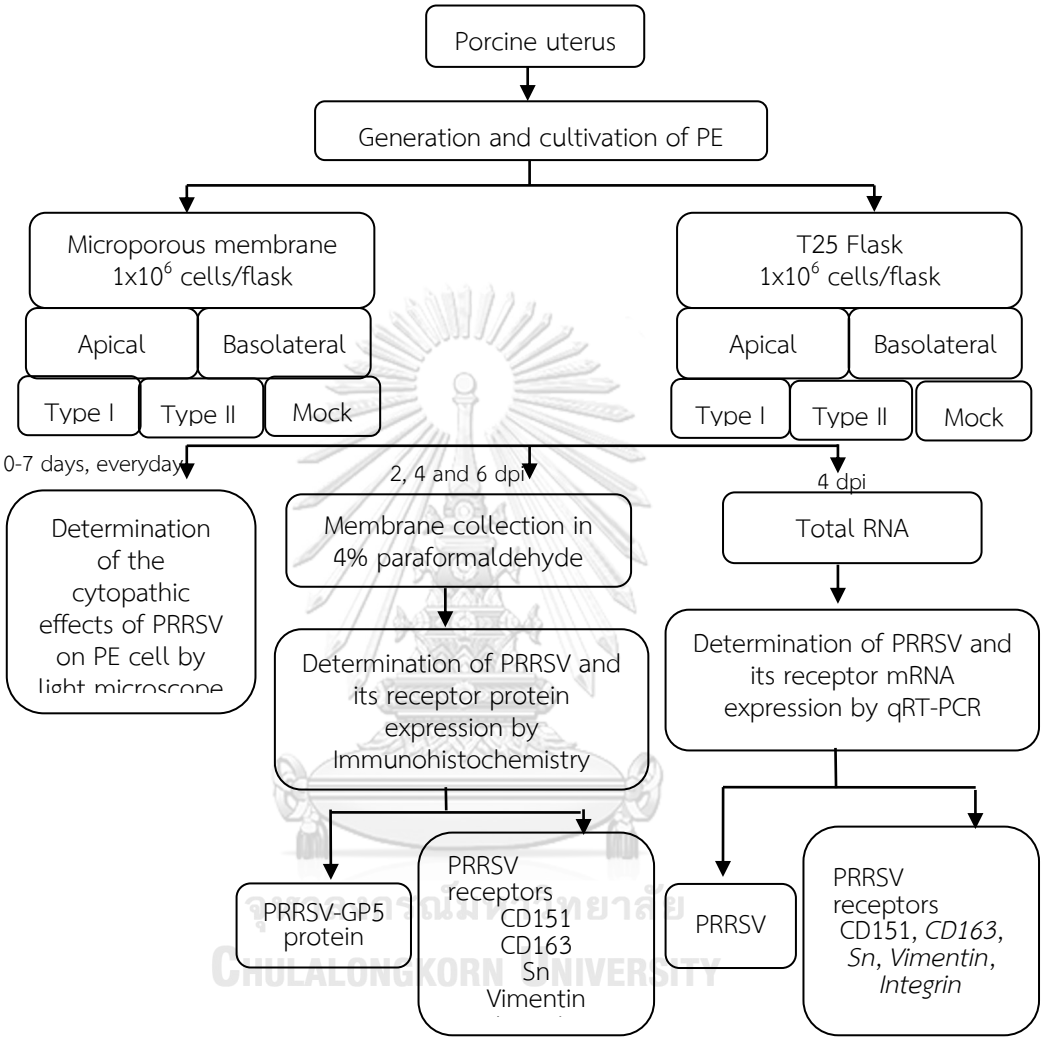
PART I: To evaluate the cellular responses of PE cells to PRRSV infection in the endometrium regarding to the epithelial histology and the modification of PRRSV receptor expression related to viral replication in the different genotypes and routes of infection

To evaluate the cellular responses of PE cells to PRRSV infection in the endometrium, mRNA and protein expression of PRRSV and its receptor was performed.

3.6 Determination of microscopic changes

PE cells grown in microporous membrane were observed microscopic changes routinely during 0-6 dpi under inverted light microscope (CK30-F200, Olympus, Tokyo, Japan). Changes in cellular morphology or CPE was recorded and the photograph taken by microscope digital camera (BX50F and UC50, Olympus, Tokyo, Japan). Common CPE consists of total destruction, subtotal destruction, focal degeneration, swelling and clumping, foamy degeneration, syncytium and inclusion bodies (Albrecht et al., 1996). The pixels of area demonstrating CPE were triplicate measurement and compared to those of total area of cell confluent by using Adobe Photoshop®. The presence of CPE was calculated as the percentage of the total CPE per area (mm^2). Microscopic changes were compared among different groups of PRRSV infection and between apical and basolateral side of infection (n=5 pigs).

Experimental design: Part I



3.7 Determination of PRRS receptor mRNA expression

3.7.1 Total RNA isolation and cDNA synthesis

Total RNA was extracted from mock or PRRSV infected PE cells at 4 dpi ($1x10^6$ cells) cultivated in T25 flask using TRIzol® reagent (Invitrogen™, CA, USA). According to 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. Chloroform 40 µl was added and centrifuged at 12,000 *g*, 4°C

for 15 min (Micro Centaur Plus, MSE, London, UK) to separate nucleic acid from contaminant. Total RNA was collected from the transparent layer of sample and precipitated in 100 µl of isopropanol. The RNA pellet was collected after centrifugation and washed with 75% ethanol in diethylpyrocarbonate (DEPC). The final total RNA pellets were air dried and dissolved 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 Fisher Scientific, MA, USA), and purity was determined by calculation of the OD_{260}/OD_{280} ratio. The RNA sample was accepted when the ratio is between 1.8 and 2.0.

The first strand DNA was synthesized by reverse transcription using cDNA synthesis kit (iScriptTM, Bio-rad, Inc., CA, USA). According to the manufacturer's protocol, total RNA 3 µg was mixed with 20 µl of cDNA synthesis reaction containing 2 µl Oligo dT primer, 4 µl 5x iScript reaction mix, 1 µl iScript reverse transcriptase and nuclease-free water. The reaction was transformed to cDNA using TGradient thermocycler (Biometra, Germany) using the following cycle 25°C 3 min, 46°C 20 min, and 95°C 1 min. The cDNA product was stored at -20°C until performing real-time RT-qPCR.

3.7.3 Determination of PRRSV receptor mRNA expression

The mRNA expression of PRRSV mediators was investigated by real-time PCR using a SYBR green based qPCR kit (GeneOn, Deutschland, Germany). Following the manufacturer's protocol, 3 µg of cDNA template was mixed in qPCR SYBR mastermix in the presence of forward and reverse primers for *CD151*, *CD163*, *Sn*, *Integrin* or *Vimentin* gene. The following program: 95°C for 3 min to activate the reaction, followed by 40 cycles of amplification steps including denaturation at 95°C 20 sec, annealing at 60°C 30 sec and extension at 72°C 30 sec respectively. The amplification products were confirmed the specificity by performing 1.5% agarose gel electrophoresis and melting curve analysis. During the amplification, the numbers of cycle initially detecting the emission of SYBR green that incorporated into PCR product of each sample were recorded and reported as threshold cycle (C_t).

Expression of each interested gene was calculated by normalizing with the C_t of *GAPDH* (ΔC_t), by using following equation.

$$\Delta C_t = C_t \text{ of interested gene} - C_t \text{ of GAPDH gene}$$

The modification of PRRSV mediator mRNA expression by PRRSV was demonstrated as fold changes using $2^{-\Delta\Delta Ct}$ equation (Livak and Schmittgen, 2001). The $\Delta\Delta C_t$ of each group was calculated by following equation (n = 5 pigs).

$$\Delta\Delta C_t = \Delta C_t \text{ of PRRSV inoculation} - \Delta C_t \text{ of Mock}$$

In this study, all primer sets shown in Table 3 were designed by iSciencetech (iScience technology, BKK, Thailand) using the NCBI databases for the available porcine sequences of PRRSV mediator genes.

Table 3 Sequences of PRRS receptor specific primer sets

Gene	Primer sequences (5' —> 3')	Accession number	Product size (bp)
CD151	F: TGTGTGCAGGTGTTCGGCAT R: TCAGCGCATCCTGAGAAGCT	NM_001243865.1	123
CD163	F: AATTCCAGTGTGAGGGGCAC R: AGCGGATTTGTGTGTATCTTGAG	HM991330.1	123
Integrin	F: GACCAGGTGACCCGTTTCAA R: TCCAGCCAATCTTCTCGTCAC	NM_214002.1	124
Sn	F: CCCAAACCTCAGGACCTCAG R: GTCCAGCTCCTCTCGGTTCTT	EU131884.1	87
Vimentin	F: TCCAAGTTTGCCGACCTCTC R: GACTCGTTGGTCCCCTTGAG	XM_005668107.1	140
GADPH	F: GGACCAGGTTGTGTCCTGTGA R: TCCACCACCCTGTTGCTGTAG	NM_001206359.1	143

3.8 Determination of PRRSV receptor protein expressions

To observe the cellular protein expressions of PRRSV receptors including CD151, CD163, Sn, Integrin and vimentin, immunohistochemistry was performed at 2, 4, 6 dpi. PRRSV infected cells on supporting membrane were fixed with 4% paraformaldehyde in PBS, pH 7.4 for 10 min at 25°C and processed for immunohistochemistry as described previously (Deachapunya and O'Grady, 1998). The collected membranes were washed three times with 0.05% tween in PBS (PBST) prior to blocking endogenous peroxidase with 10% H_2O_2 in methanol. The non-specific antibody was blocked using 2% horse serum in PBST. To observe the expression of PRRSV protein and PRRSV receptors, the membrane was incubated overnight with primary antibodies as shown in Table 4. Some samples were incubated with the antibody diluent for the negative control. The sample then was washed and incubated with an appropriate secondary antibody shown in Table 4. Dilution of antibodies were optimized following product's instructions. After removal of excessive antibodies and washing with PBS, the avidin-biotin-enzyme complex (Vector Labs, Burlingame, CA) was added and incubated for 30 min at 25°C. DAB (3,3-diaminobenzidine tetrahydrochloride) was used as substrate for staining. Counter staining with hematoxylin was also performed. The immunoreactive PE cells were quantified under light microscope with magnification of 20X. (BX50F and UC50, Olympus, Tokyo, Japan). Using the Image-Pro Plus 4.5 software (Media Cybernetics, Silver Spring, EUA), the total area of immunoreactive staining (dark-brown staining) was measured and expressed in the pixel numbers for the positive results. Expressions of PRRSV and PRRSV receptors were calculated and reported as % immunoreactivity/field (n=5 pigs). Additionally, the PRRSV-GP5 positive results in all inoculated experiments of PE cells were confirmed by comparing to the immunoreactive positive cell of PRRSV infected MARC-145. Presence of CPE was also observed corresponding to PRRSV positive cells in the same field of observation.

Table 4 Antibodies for determination of PRRSV and PRRSV mediator cellular protein expression

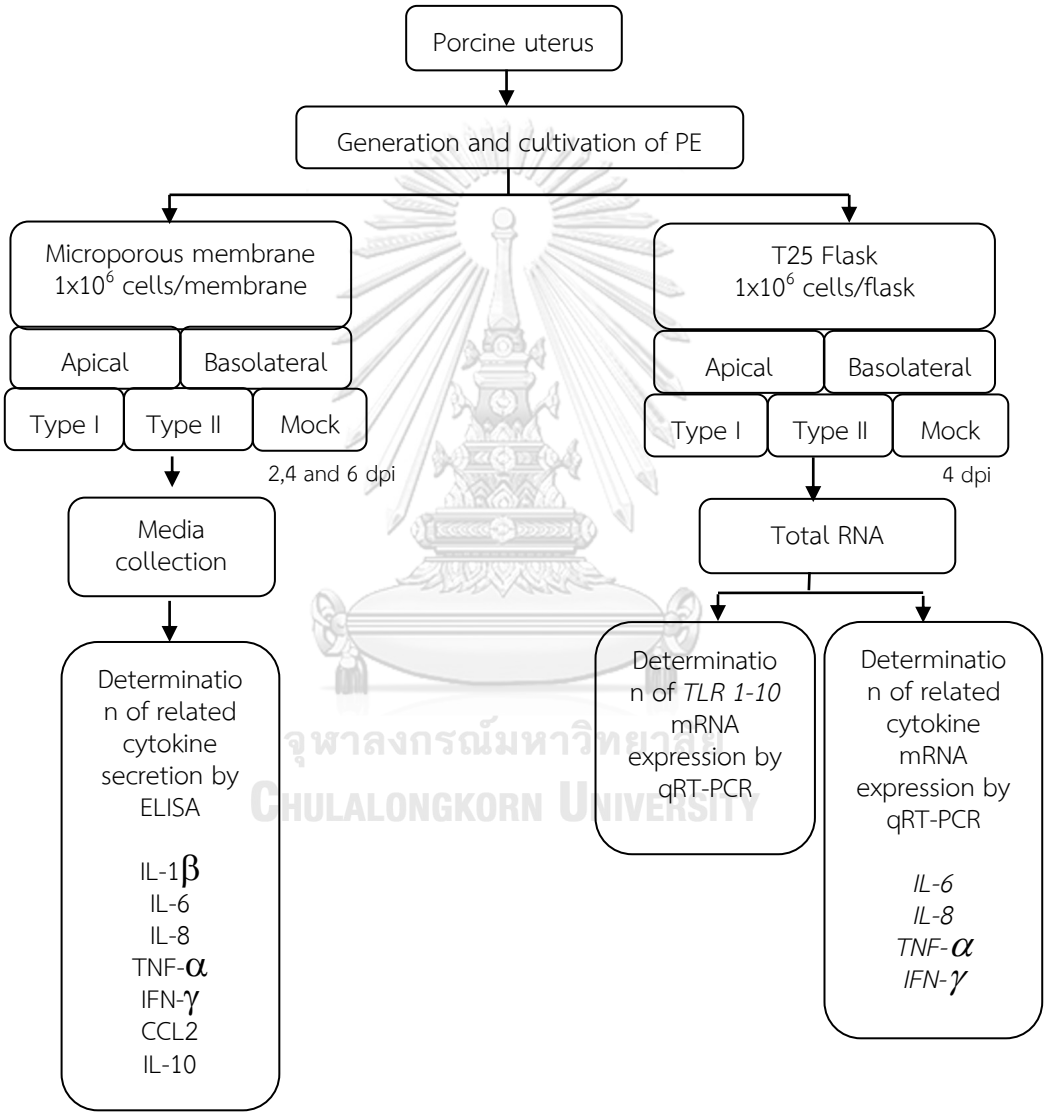
Primary antibodies	Dilution	Secondary antibodies	Dilution
Goat-anti-CD151	1:25	Biotinylated donkey-anti-goat IgG	1:2000
Goat-anti-CD163	1:25	Biotinylated donkey-anti-goat IgG	1:2000
Goat-anti-integrin	1:250	Biotinylated donkey-anti-goat IgG	1:2000
Mouse-anti-sialoadhesin	1:25	Universal Anti-Mouse/Rabbit IgG	1:2000
Mouse-anti-vimentin	1:250	Universal Anti-Mouse/Rabbit IgG	1:2000
Rabbit-anti-PRRSV-GP5	1:100	Universal Anti-Mouse/Rabbit IgG	1:2000

3.9 Statistical analyses

All data from at least five different primary cultured PE cells isolated from 5 pigs were expressed as mean±SEM. Statistical analyses were done using analysis of variance (ANOVA) to compare the differences between mock and infected group on the gene expression at the one specific time (4 dpi). The experiment observed at many time points to evaluate the effects of PRRSV different genotype/route of infection were analyzed by two-way repeated measure ANOVA. Post-hoc test was additionally performed with the Dunnett's test to compare the differences from control or using the Newman-Keuls and Bonferroni test to compare the differences between two groups. A *P* value less than *0.05* was considered as significant difference between two groups. Graphpad Prism 5.0 (Graphpad software Inc., CA, USA) was used to perform for all statistical analyses.

PART II: To evaluate the mucosal innate immunological system responses of PE cells to PRRSV infection in the endometrium regarding to TLRs expression and related cytokines synthesis and release in the different genotypes and route of infection

Experimental design: Part II



3.10 Determination of *TLRs* mRNA expressions

The protocol was similar to the method of the determination of PRRSV receptor mRNA expressions in part I. The mRNA expression of TLRs reported as fold change normalized with *GAPDH* was calculated by $2^{-\Delta\Delta Ct}$ equation (Livak and Schmittgen, 2001), but the specific primer sets of porcine *TLRs* designed by iSciencetech (iScience technology, BKK, Thailand) as shown in table 5 were used instead.

Table 5 Sequences of porcine TLRs specific primer sets

Gene	Primer sequences (5' —> 3')	Accession number	Product size (bp)
TLR1	F: CACAGAGTCTGCACATTGTTTATCC R: GATTTACTGCGGTGCTGACTGA	NM_001031775.1	81
TLR2	F: GTGCTTTCCGAGAACTTTGT R: GCAGAATGAGGATGGCG	KF460452.1	106
TLR3	F: TCCAACTAACAAACCAGGC R: ACATCCTTCCACCATCT	NM_001097444.1	186
TLR4	F: AAGGTTATTGTCGTGGTGT R: CTGCTGAGAAGGCGATAC	NM_001293316.1	179
TLR5	F: TTGCATCCAGATGCTTTTCA R: TTCAACTTCCCAAATGAAGGA	XM_012506471.1	182
TLR6	F: TCACCTCTCTGACATCAGCTTTCT R: TGATATCAAGGCACTGCATCCT	NM_213660.1	80
TLR7	F: GGACCATCTGGTAGAGATCGATTT R: TTCTGGTGCACAGGTTGTCTTT	NM_001097434.1	80
TLR8	F: CCGCACTTCGCTATCTAAAC R: GAAAGCAGCGTCATCATCAA	NM_214187.1	791
TLR9	F: AGATGTTTGCTCGCCT R: GGACACTCGGCTATGGA	KC860785.1	308
TLR10	F: CTACCAGGTATCCTGCACTGAAAG R: GGCAACATTTACGCCTATCCTT	NM_001030534.1	81

3.11 Measurement of cytokine secretion

To determine the innate immune response of PE cells mediated by TLRs, the concentration of cytokines, including CCL-2, IL-1β, IL-6, IL-8, IL-10, IFN-γ and TNF-α, were investigated by enzyme linked immunosorbent assay (ELISA). Media collected form apical and basolateral compartment of each experimental group were used as sample for ELISA by using ELISA kit (Duoset®, R&D system, MN, USA) following the manufacturer's protocol (n=5 pigs). Briefly, 96-well-plate was coated with 100 µl of capture antibody in PBS at 4°C overnight. Non-specific antibody was blocked with 100 µl of 1% BSA in PBS for 1 hr at room temperature followed by adding standard or sample. Concentrations of standard were prepared according to the manufacturer's guideline. After incubation with sample, HRP-conjugated secondary antibody was added into each well at 100 µl and incubated for 30 min at room temperature. Adding the 50 µl of TMB substrate was performed and incubated for 15 min. To stop the reaction, 50 µl of 2M H_2SO_4 was added into each well. The reaction was read OD at 450 nm/620nm using a microplate reader (Epoch, Biotek, VM, USA). Concentrations of CCL-2, IL-1β IL-6, IL-8, IL-10, IFN-γ and TNF-α in the samples were calculated by comparing OD the standard curve. The data of cytokine concentration by each date were summed up and reported as accumulated cytokine secretion.

3.12 Determination of mRNA expression of IL-6, IL-8, IFN-γ and TNF-α

The protocol was similar to the method of the determination of PRRSV receptor mRNA expressions in part I. The mRNA expression of TLRs reported as fold change normalized with *GAPDH* was calculated by $2^{-\Delta\Delta Ct}$ equation (Livak and Schmittgen, 2001) but the specific primer sets of porcine cytokines designed by iSciencetech (iScience technology, BKK, Thailand) as shown in Table 6 were used instead.

Table 6 Sequences of porcine cytokine specific primer sets

Gene	Primer sequences (5' —> 3')	Accession number	Product size (bp)
IL-6	F: AGATGCCAAAGGTGATGCCA R: ACAAGACCGGTGGTGATTCTCA	NM_214399	257
IL-8	F: TTTCTGCAGCTCCTCTGTGAGG R: CTGCTGTTGTTGTTGCTTCTC	M99367 M86923	269
IFN-γ	F: GTTTTTCTGGCTCTTACTGC R: CCTCCGCTTTCTTAGGTTAG	X53085	410
TNF-α	F: ATCGGCCCCCAGAAGGAAGAG R: GATGGCAGAGAGGAGGTTGAC	M29079 X54859	351

3.13 Statistical analyses

All of data were shown as mean±SEM. Statistical analyses were performed using Graphpad Prism 5.0 (Graphpad, CA, USA). To determine the significant differences among mean values of mRNA expressions of TLRs, IL-6, IL-8, IFN-γ, TNF-α and accumulated cytokine secretion one-way ANOVA was tested and followed by post-hoc test with the Newman-Keul. Significant differences were considered at 95% degree of freedom ($P<0.05$).

PART III: To evaluate the susceptibility of PRRSV re-infection in the PE cells primary infected with PRRSV regarding to the cellular response and mucosal innate immunological system responses

To demonstrate the cellular adaptation, to the subsequent PRRSV infection. The primary infected PE cells at 4 dpi were re-infected with the same genotype and side of the earlier PRRSV infection. The mRNA expression of TLRs and related cytokine, occurrence of CPE, cellular expression of PRRSV-GP5 and accumulated cytokine concentrations were determined to evaluate the cellular responses of PE cells after PRRSV re-infection.

3.14 PRRSV infection of PE cells

PE cells were plated into T25 flask for collecting total RNA or cultured in monolayer PE cells using microporous membrane for determining the differences between apical/basolateral infections. PE cells were grown in 5% CO_2 at 37^{o}C with maintaining medium. At 90% confluent of PE cells in a T25 flask or at 7 days after cultivation in microporous membrane, PE cells were infected with PRRSV following the protocol as previously described. In addition, PE cells in microporous membrane were infected at apical or basolateral side with PRRSV type I or type II according to previous protocol. The media were refreshed every 48 hr, and the appearance of CPE was observed.

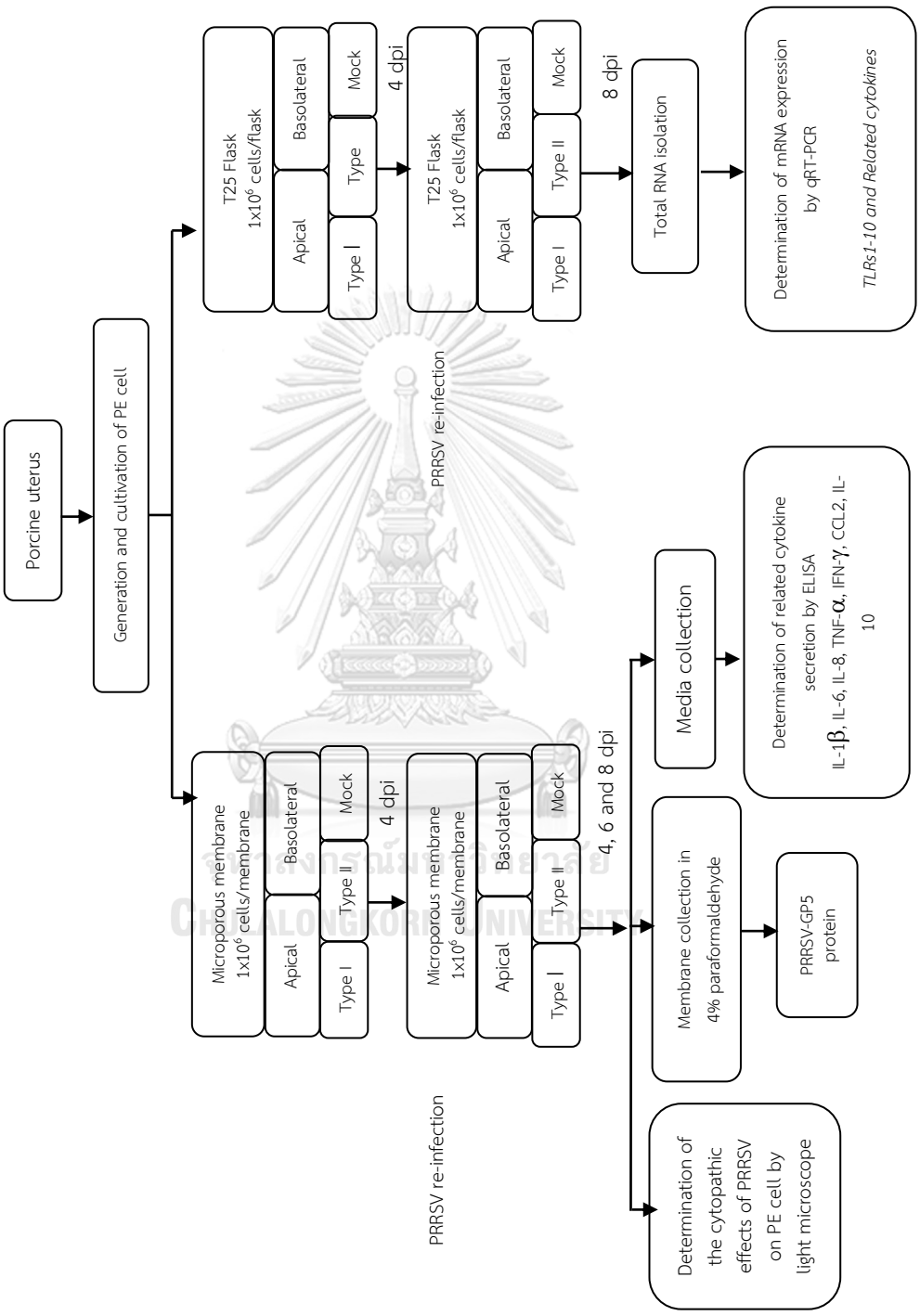
At 4 dpi, all of PE cells were re-infected with the same previous condition. Infection was performed by the homologous genotype at the same route that had been previously infected. Mock re-infection was performed by incubating mock infected PE cells with PRRSV-free DMEM. During 4, 6 and 8 dpi, media bathing apical and basolateral sides were collected into micro-centrifuge tube containing cocktail-protease inhibitor at the same volume as a sample and kept at -20^{o}C until subsequent analysis). The PE cells on microporous membranes were fixed in 4% paraformaldehyde at 4, 6 and 8 dpi to determine cellular expression of PRRSV-GP5 using immunohistochemistry. Total RNA was collected at 8 dpi to subsequently

perform qPCR for determining *TLRs 1-10* and related cytokine mRNA expressions from infected or re-infected groups as appropriate.

3.15 Statistical analyses

All of data were shown as mean±SEM. Statistical analyses were performed using Graphpad Prism 5.0 (Graphpad, CA, USA). To determine the significant differences among experimental groups, mRNA expression of all interested genes and accumulated cytokine secretion were analyzed using to one-way ANOVA with followed by the Newman-Keul post-hoc test. Two-way ANOVA was used to analyze cellular expression of PRRSV-GP5 and occurrence of CPE. Bonferroni post-hoc test was done following two-way ANOVA analysis. Significant difference was considered at 95% degree of freedom ($P<0.05$).

Experimental design: Part III



CHAPTER IV
RESULTS

PART I: To evaluate the cellular responses of PE cells to PRRSV infection in the endometrium regarding to the epithelial histology and the modification of PRRSV receptor expression related to viral replication in the different genotypes and routes of infection

Determination of cytopathic effects and morphologic changes of PE cells in response to PRRSV infection

To determine the susceptibility of PE cells to PRRSV infection, the cell morphological changes following viral inoculation were daily observed. Exposure of PE cell monolayer to the PRRSV-isolated solution for 1 hr demonstrated microscopic changes of CPE and positive PRRSV immunoreactivity at 4 dpi (Fig. 8). Both morphological changes and PRRSV immunoreactivity was absence or less detected in mock-treated PE cells at 4 dpi (Fig. 8A and 8B). By contrast, all infected PE cells revealed CPE which was observed as early as 2 dpi and remained up to 6 dpi. The PRRSV-induced CPE was demonstrated as vacuolization, syncytial formation (Fig. 8C) or plaques (Fig. 8D). Dissemination of vacuolated cells reflecting focal degeneration was generalized in infected PE cells at 2-6 dpi.

The percentage of observed CPE area in PE cells induced by PRRSV at different genotypes and routes of infection at 2, 4 and 6 dpi were compared in figure 9. In mock-infected groups, the % CPE was not detected at 2 dpi, but increased at 4 and 6 dpi. The overall CPE caused by apical infection of either PRRSV type I or type II (18.84±3.47%) was greater than those induced by basolateral infection (6.13±1.04%, $p<0.05$). When apically infected, type II produced 20-40% of CPE which was higher than type I at 4 and 6 dpi ($p<0.05$, Fig. 9). However, at 6 dpi, generalized focal degeneration found in apically type II-infected groups was not different from those found in mock-infected group. Likewise, basolateral infection of type II produced the CPE area (<20%) higher than those of type I (<10%) at 2 dpi ($p<0.05$) while no

difference in CPE between type I and II infection was observed at day 4 and 6 post infection (Fig. 9).

Determination of the cellular PRRSV following the PRRSV infection in PE cells

To determine viral entry and persistence in infected PE cells, the expression of PRRSV was identified using the antibody specific to viral structural protein PRRSV-GP5 by immunohistochemistry. As shown in figure 8, the PRRSV-GP5 immunoreactivity was not detected in mock-treated cells (Fig. 8B), whereas it was detected in all PE cells infected with PRRSV, particularly at surrounding area of CPE at 4 dpi (Fig. 8D). Cell release of PRRSV was additionally confirmed by incubating MARC-145 cells with the media collected from PRRSV-infected PE cells and evaluating of PRRSV-GP5 protein at 4 dpi (Fig. 8E-8F). As positive control, the PRRSV-GP5 protein was completely detected in MARC-145 cells incubated with PRRSV isolated from infected lung (Fig. 8E). The CPE was more obviously observed in MARC-145 cells infected with PRRSV isolated from lung than that secreted from infected PE cells (Fig. 8F).

The immunoreactivity of PRRSV-positive PE cells observed at 2, 4 and 6 dpi were shown in figure 10. Although the CPE was observed in 4 and 6 dpi, the PRRSV-GP5 immunoreactivity was not detected in mock-infected cells at any day post infection. The immunoreactivity up to 50% per field was detected in the apical PRRSV infection with either type I or type II during 2-6 dpi. At 2 and 4 dpi, the apical infection of PRRSV type II expressed PRRSV positive cells more than type I ($p<0.05$, Fig. 10), whereas both types produced comparable PRRSV immunoreactivity at 6 dpi (Fig.10, $p>0.05$). In contrast to apical infection, basolateral infection with only type II increased the PRRSV immunoreactivity by 40% during 2-4 dpi while the PRRSV-positive cells could not be detected in PE cells within 2-6 dpi following basolateral PRRSV infection (Fig. 10).

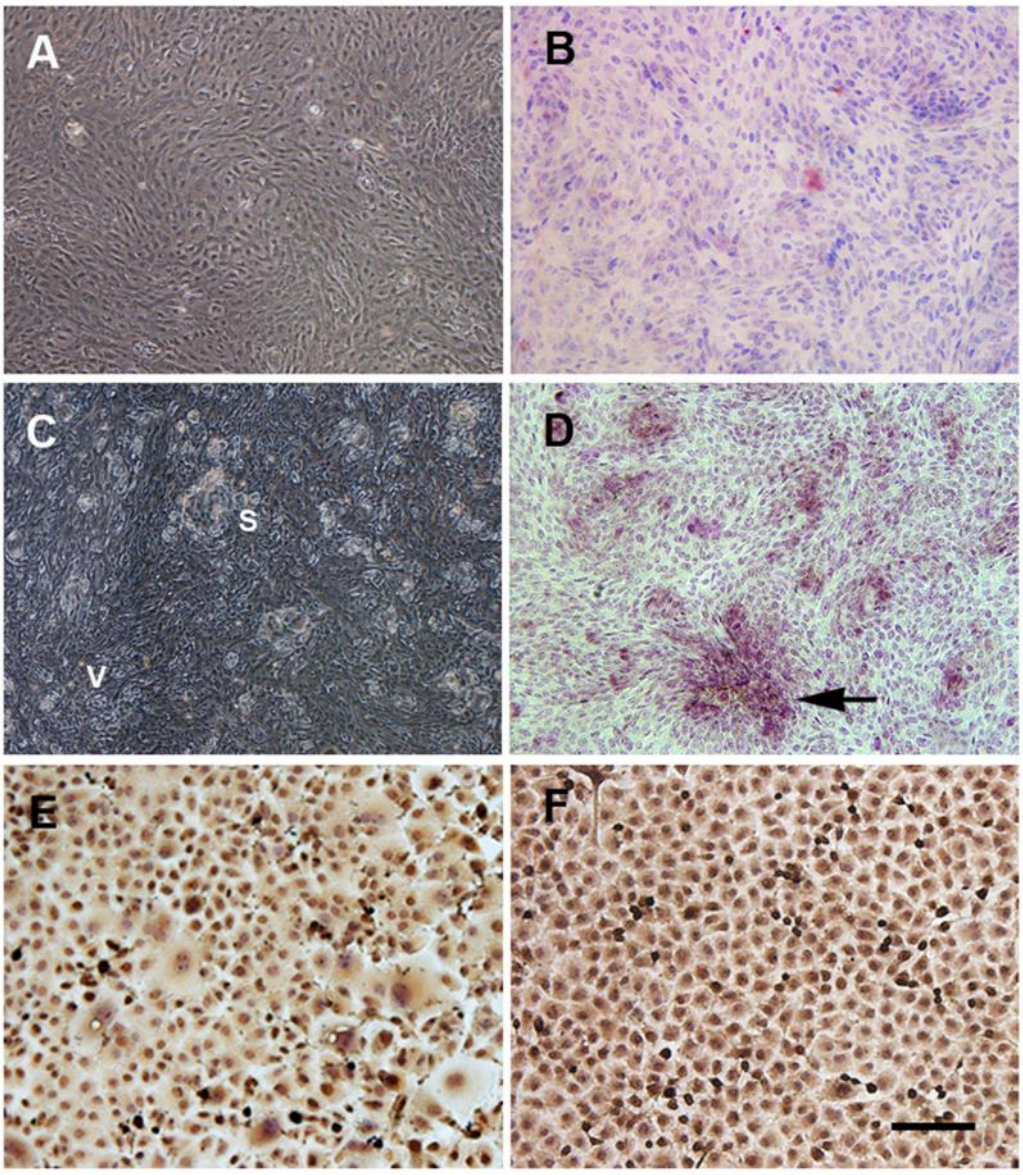


Figure 8 The cytopathic effects and intracellular PRRSV observed in PE cells and MARC-*145* cells at *4* dpi under a light microscope (n=*5* pigs). (A and C) Micrograph respectively represents mock and infected PE cells at *4* dpi. (B, D, E and F) Immunohistochemistry using PRRSV-GP*5* antibody respectively observed in mock PE, lung isolated PRRSV infected-PE, lung isolated PRRSV infected-MARC-*145* and infected PE media infected-MARC-*145* cells at *4* dpi. Vacuolization (v) and syncytial formation (s) are shown. Horizontal arrow represents cellular aggregation (plaques). The dark-brown color demonstrates PRRSV-GP*5* immunoreactivity. Scale bar = *100* µm.

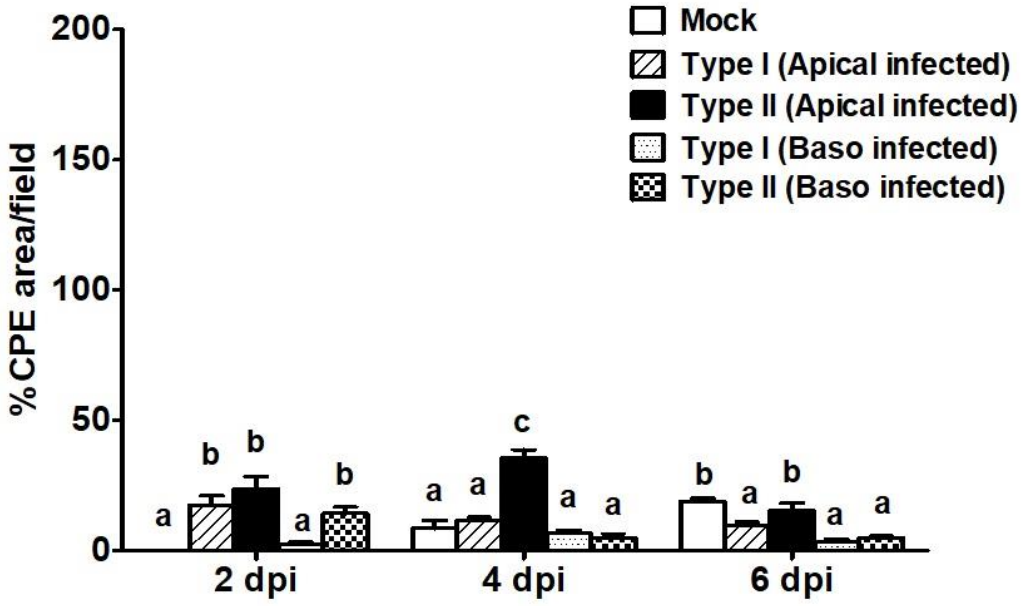


Figure 9 Effects of PRRSV infection on producing cytopathic effects in PE cells. The microporous membrane-grown PE cells were infected via apical or basolateral route with mock, PRRSV type I or type II for 1 hr. The area of CPE was observed by light microscope and measured at 2, 4 or 6 dpi. Bar graph represents mean ± SEM of % CPE area per field (n=5 pigs). Bar graph with different letters (a, b) indicates significantly different at $p < 0.05$ by two-way ANOVA followed by Bonferroni post-hoc test.

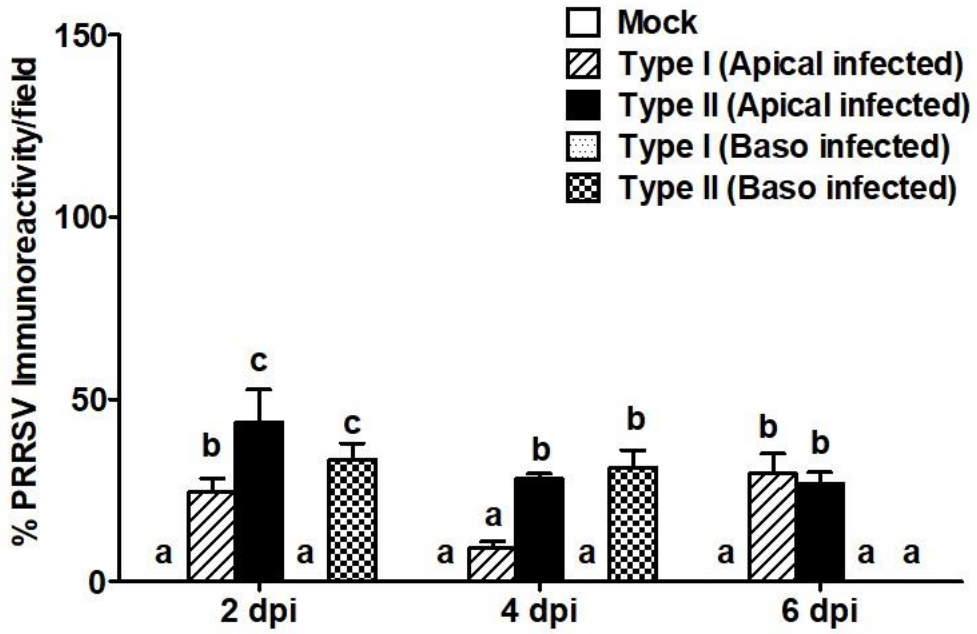


Figure 10 Cellular expression of PRRSV-GP5 protein in PE cells. The microporous membrane-grown PE cells were infected via apical or basolateral route with mock, PRRSV type I or type II for 1 hr. PRRSV protein were evaluated by immunohistochemistry using antibody against PRRSV-GP5 and observed under light microscope at 2, 4 or 6 dpi. Bar graph represents mean ± SEM of % immunoreactive area per field (n=5 pigs). Bar graph with different letters (a, b, c) indicates significantly different at $p < 0.05$ by two-way ANOVA followed by Bonferroni post-hoc test.

Effects of PRRSV infection on the PRRSV mediator gene expression in PE cells

The mRNA expression of the putative PRRSV mediators required for the susceptibility of PE cells to PRRSV was determined by RT-qPCR (Fig. 11). Prior to PPRSV infection (0 dpi), PE cells expressed low levels of *CD151* (0.008±0.001), *CD163* (0.007±0.002), *Sn* (0.40±0.04), *integrin* (0.002±0.0002) and, to a greater extent, *vimentin* (3.11±0.86) in relative to *GAPDH*. These gene expressions of the non-infected cells were not different from those of mock-treated cells. Exposure to

PRRSV type I significantly up-regulated *CD151, CD163, Sn* and *integrin* at 4 dpi as compared to the mock-treated groups (*p<0.05*, Fig. 11). Infection with PRRSV type II was only found to up-regulate *CD163* in which its expression was significantly lower as compared to type I infection (*p<0.05*). Moreover, there was no significant difference in vimentin expression among groups.

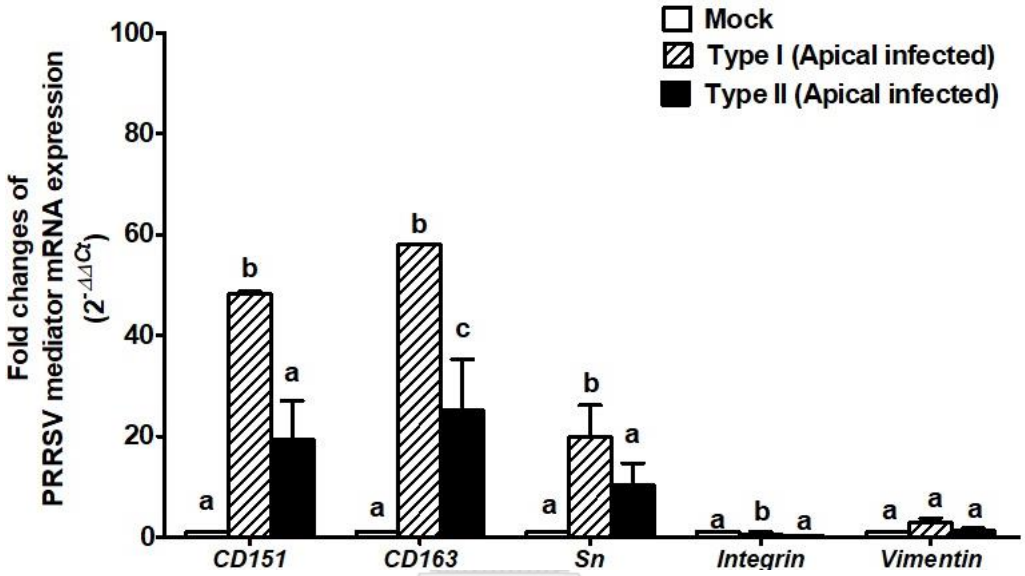


Figure 11 Effects of PRRSV infection on mRNA expression of PRRSV mediators in PE cells. PE cultured in T25 flask were infected with mock, PRRSV type I or type II for 1 hr. Total RNA was isolated at 4 dpi for determining of *CD151, CD163, Sn, integrin* and *vimentin* normalized to house-keeping gene *GAPDH* by qPCR. Bar graphs represent mean ± SEM of the fold changes of PRRSV mediator mRNA expression from mock using the $2^{-\Delta\Delta Ct}$ (n=5 pigs). Bar graph with different letters (a, b) indicates significantly different at *p<0.05* by one-way ANOVA followed by Newman-Kuel post-hoc test.

Effects of PRRSV infection on the cellular expression of PRRSV mediators in PE cells

Protein expressions of PRRSV mediators in PE cells were further evaluated by immunohistochemistry. Before infection at 0 dpi, immunoreactivity of some PRRSV mediators including, CD151, Sn, integrin and vimentin were differentially expressed by PE cells, whereas the expression of CD163 could not be detected (Fig. 12). The immunoreactive CD151 or Sn was distributed in the cytoplasm, but the intensity and positive cells of CD151 were higher than those of Sn. On the other hand, the expression of vimentin and integrin was revealed at the adjunction of cells but not in the cytoplasm. In particular, the vimentin immunoreactivity had the characteristic of fiber-like shape (Fig. 12E).

Cellular expression of PRRSV mediators observed during 0-6 dpi was analyzed by comparing the differences in genotypes and sites of infection (Table 7). As compared to mock, apical infection by PRRSV type I up-regulated CD163 at 2 and 4 dpi ($p<0.05$), and integrin at 2 dpi ($p<0.05$). PE cells apically infected with PRRSV type II up-regulated CD151 at 6 dpi ($p<0.05$), CD163 at 2 and 4 dpi ($p<0.05$), integrin at 2, 4 and 6 dpi ($p<0.05$) and vimentin at 4 dpi ($p<0.05$). However, down-regulation of PRRSV mediators, including Sn (at 4 dpi), integrin (at 4 dpi) and vimentin (at 4 and 6 dpi) were caused only by apical infection with PRRSV type I ($p<0.05$).

In contrast to apical infection, basolateral infection by PRRSV type I increased cellular expression of CD151 at 6 dpi ($p<0.05$). Both PRRSV type I and II infection at the basolateral side up-regulated CD163 at 2 and 6 ($p<0.05$), Sn at 4 dpi and 6 dpi ($p<0.05$). Down-regulated Sn at 2 dpi ($p<0.05$) was observed with basolateral type I infection while integrin was down-regulated by both type I and type II infection at 2 and 6 dpi ($p<0.05$). Vimentin was also down-regulated by type I infection via basolateral surface at 2 and 4 dpi, and by type II at 6 dpi.

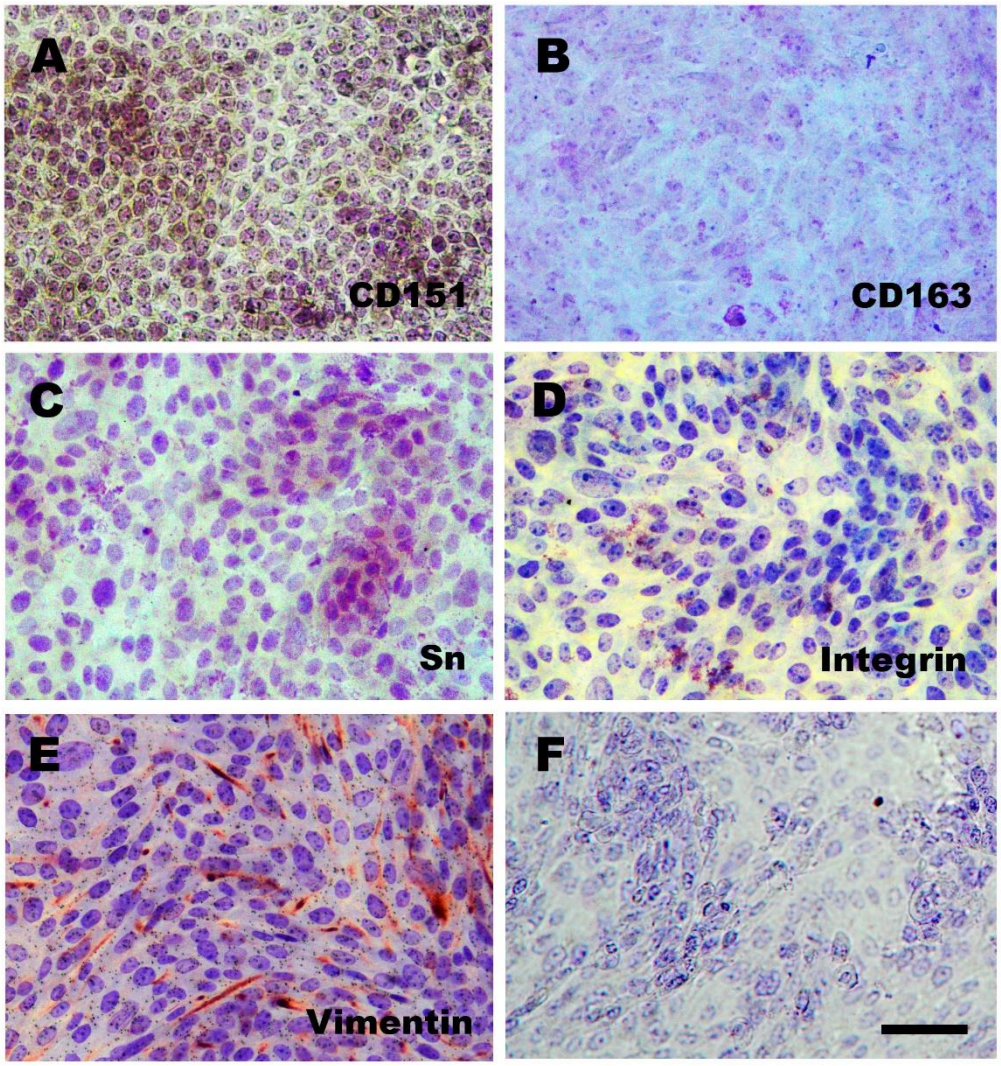


Figure 12 Cellular expression of PRRSV mediator proteins in non-infected PE cells. The Immunohistochemistry staining was performed in microporous membrane-grown PE cells prior to PRRSV infection (at 0 dpi) with antibodies against (A) CD151, (B) CD163, (C) Sn, (D) integrin, or (E) vimentin. (F) Primary antibody omitted negative control was performed in each experiment. The dark-brown color representing positive immunoreactivity of each mediator protein was observed under light microscope. Scale bar = 100 μm.

Table 7 Effects of PRRSV infection on cellular expression of PRRSV mediator proteins in PE cells. The cells cultured in microporous membrane were apically or basolaterally infected with mock, PRRSV type I or type II and determined for protein expression of CD151, CD151, CD163, Sn, integrin and vimentin using immunocytochemistry at 0 (before infection), 2, 4 and 6 dpi. All data are mean ± SEM (n=5 pigs) of the percentage of immunoreactive area per field. Different letters (a, b, c, d) indicate significantly different at each time point at $p<0.05$ by two-way ANOVA followed by Bonferroni post-hoc test.

dpi	Mock	*Apical Infection*		*Basolateral Infection*	
		Type I	Type II	Type I	Type II
CD 151					
0 dpi	42.21± 3.45^{a}	42.21±1.94^{a}	42.21±3.45^{a}	42.21±3.45^{a}	42.21±3.45^{a}
2 dpi	42.87± 6.39^{a,b}	65.60±4.30^{b}	46.91±3.12^{a,b}	43.30±1.02^{a}	46.36±12.90^{a,b}
4 dpi	41.55±4.25^{a,b}	44.02±2.40^{a,b}	35.35±5.53^{b}	55.38±12.77^{a}	43.74±13.09^{a,b}
6 dpi	41.60±4.89^{a}	46.54±1.57^{a}	72.41±3.92^{b}	83.46±7.4^{b}	60.83±4.92^{a,b}
CD 163					
0 dpi	1.01±0.27^{a}	1.01±0.27^{a}	1.01±0.27^{a}	1.01±0.27^{a}	1.01±0.27^{a}
2 dpi	0.61±0.21^{a}	15.60±4.30^{b}	30.34±4.32^{c}	24.02±9.77^{b,c}	15.77±12.90^{b}
4 dpi	0.42±0.16^{a}	22.31±4.28^{b}	21.72±1.65^{b}	9.37±0.63^{a}	4.10±0.66^{a}
6 dpi	0.40±0.18^{a,b}	3.64±1.57^{a,b}	8.12±0.72^{a,b}	0.66±0.31^{b}	5.98±0.85^{a,b}
Sn					
0 dpi	22.56±0.12^{a}	22.56±0.12^{a}	22.56±0.12^{a}	22.56±0.12^{a}	22.56±0.12^{a}
2 dpi	22.04±0.07^{a,b}	28.26±2.92^{a}	16.11±3.74^{b,c}	9.98±1.80^{c}	39.54±7.55^{d}
4 dpi	22.73±0.08^{a}	0.14±0.015^{b}	14.37±3.44^{a}	49.98±2.32^{c}	47.56±5.79^{c}
6 dpi	24.19±0.52^{a,b}	21.05±3.36^{a}	15.21±2.75^{a}	28.95±5.27^{a,b}	35.64±4.80^{b}
Integrin					
0 dpi	19.32±2.19^{a}	19.32±2.19^{a}	19.32±2.19^{a}	19.32±2.19^{a}	19.32±2.19^{a}
2 dpi	19.89±3.95^{a}	53.77±5.44^{b}	52.59±5.26^{b}	3.11±0.60^{c}	7.82±1.88^{a,c}
4 dpi	18.76±2.82^{a}	4.22±0.72^{b}	73.53±1.46^{c}	23.55±3.65^{a}	21.63±3.06^{a}
6 dpi	22.54±6.93^{a}	19.52±2.66^{a}	65.36±5.95^{b}	4.83±0.96^{c}	0.78±0.24^{c}
Vimentin					
0 dpi	32.16±3.48^{a}	32.16±3.48^{a}	32.16±3.48^{a}	32.16±3.48^{a}	32.16±3.48^{a}
2 dpi	28.89±3.70^{a}	24.56±2.17^{a,b}	17.31±1.65^{a,b}	12.95±4.10^{b}	24.90±6.73^{a,b}
4 dpi	35.44±2.74^{a}	21.98±1.10^{b}	56.88±3.42^{c}	15.54±5.78^{b}	46.71±5.18^{a,c}
6 dpi	30.85±3.54^{a}	13.32±3.68^{b}	25.09±1.77^{a,b}	29.06±6.70^{a}	11.80±1.66^{b}

The number labelled in green or red refers to significantly up- or down-regulation compared to mock, respectively ($p<0.05$).

PART II: Mucosal innate immunological responses of PE cells to PRRSV infection regarding to TLRs expression and function to release the related cytokines in the different genotypes and routes of infection

Effects of PRRSV infection on the TLRs gene expression in PE cells

The mRNA expression of TLRs response to PRRSV infection were evaluated at 4 dpi (Fig. 13). In PE cells cultured in the standard media, the confluent PE cells expressed *TLR4>TLR3>TLR1=TLR7>TLR2>TLR6>TLR10* at the ratio of 0.32±0.27; 0.10±0.01, 0.02±0.01, 0.02±0.01, 0.007±0.01, 0.001±0.001 and 0.0005±0.0001, respectively ($p<0.05$; by one-way ANOVA) to house-keeping gene *GAPDH* expression. The expression of TLR5 and TLR8 could not be detected in uninfected (mock-infected) PE cells.

Infection with PRRSV type I up-regulated *TLR1* and *TLR3,* but reduced *TLR4* expression (*p<0.05*). However, the decreased TLR4 mRNA expression by PRRSV type I observed at 4 dpi was not significantly different from the effect of type II infection (*p>0.05*). PRRSV type II but not type I up-regulated mRNA expression of TLR10 when compared to mock infection (*p<0.05*). Other TLRs, including *TLR2, TLR5, TLR6, TLR7, TLR8* and *TLR9*, were not significantly different among groups (*p>0.05*). This indicated that *TLR1, TLR3, TLR4* and *TLR10* may be the target genes of PRRSV infection**.**

Effects of PRRSV infection on the related cytokine gene expression in PE cells

At 4 dpi, gene expressions of related cytokine were observed (Fig. 14). The expression of IL-6, IL-8, IFN-**γ** and TNF-**α** mRNA were no significance among groups of infection. Type I or type II PRRSV infection did not change the mRNA expression of IL-6, IL-8, IFN-**γ** and TNF-**α** expressed by PE cells (*p>0.05*).

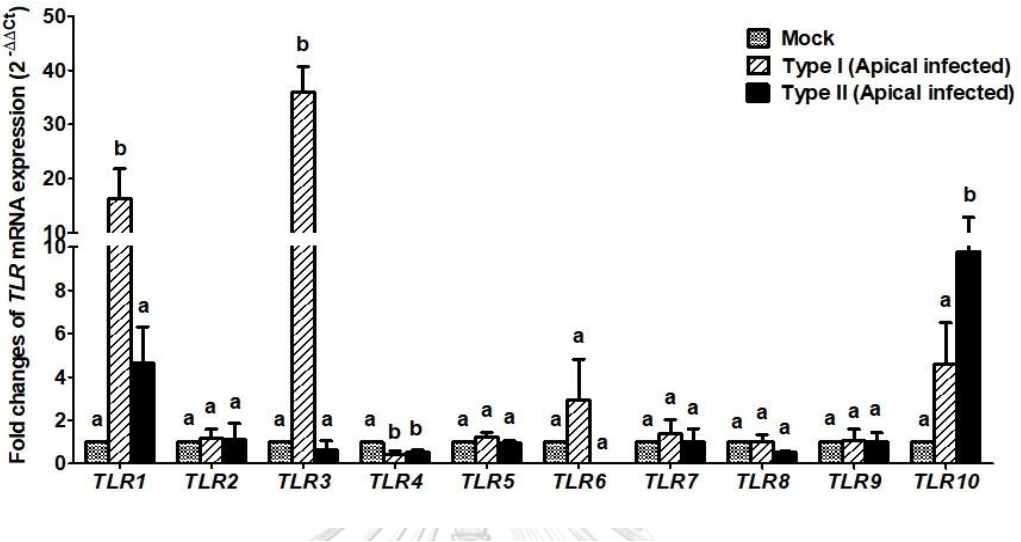


Figure 13 Effects of PRRSV infection on mRNA expression of TLRs1-10 expression in PE cells. PE cells cultured in T25 flask were infected with mock, PRRSV type I or type II for 1 hr. Total RNA was isolated at 4 dpi for determining of *TLRs1-10* normalized to house-keeping gene *GAPDH* by qPCR. Bar graphs represent mean ± SEM of the fold changes of TLRs1-10 mRNA expression from mock using the $2^{-\Delta\Delta Ct}$ (n=5 pigs). Bar graph with different letters (a, b) indicates significantly different at $p<0.05$ by one-way ANOVA followed by Newman-Kuel post-hoc test.

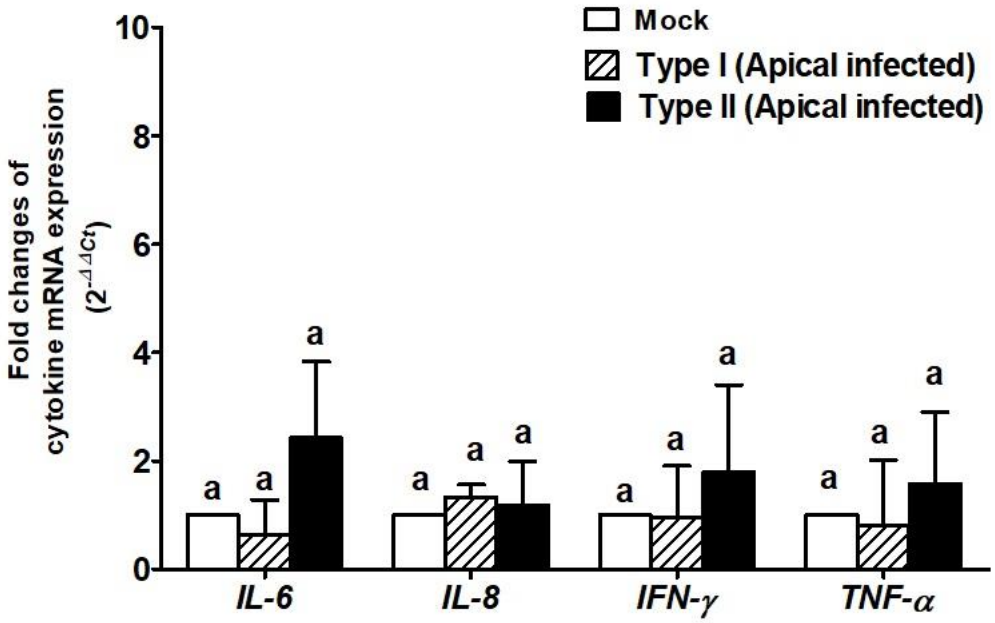


Figure 14 Effects of PRRSV infection on mRNA expression of PRRSV related cytokines in PE cells. PE cells cultured in T25 flask were infected with mock, PRRSV type I or type II for 1 hr. Total RNA was isolated at 4 dpi for determining *IL-6, IL-8, IFN-γ* and *TNF-α* normalized to house-keeping gene *GAPDH* by qPCR. Bar graphs represent mean ± SEM of the fold changes of cytokine mRNA expression from mock using the $2^{-\Delta\Delta Ct}$ (n=5 pigs). Bar graph with same letters (a) indicates no significant difference at $p>0.05$ by one-way ANOVA followed by Newman-Kuel post-hoc test.

Effects of PRRSV infection on the accumulated cytokine secretion in PE cells

To further determine the innate immunity of PE cells in response to PRRSV mediated by the TLRs signaling pathway, the related cytokines to viral infection, CCL2, IL-1β, IL-6, IL-8, IFN-γ and TNF-α were measured after PRRSV infection. The cytokines secreted by PE cells to the bathing media were collected at 0, 2, 4 and 6 dpi, and were calculated as the accumulated concentration within 6 days of observation. In the uninfected condition or mock infection, PE cells constitutively secreted CCL2, IL-1β and IFN-γ at the level below 100 pg/ml but released TNF-α and IL-6 were up to 500 pg/ml. Mostly, the accumulated IL-8 at the amount of 10 ng/ml which was 100 folds higher than other cytokines that were secreted by PE monolayer cells (Fig. 15).

As shown in figure 15, neither the amounts of CCL2, IL-1β, IL-8 or IFN-γ secretion were not affected by PRRSV infection compared to mock (*p>0.05*). Apical or basolateral PRRSV of PRRSV type I/ type II stimulated IL-6 secretion into media compartment by PE cells. However, PRRSV type II stimulated the IL-6 secretion at a significant level higher than mock and PRRSV type II (Fig. 15; *p<0.05*). In addition, all PRRSV infected PE cells decreased the secretion of TNF-α compared to mock group (*p<0.05).* However, the stimulatory effects on IL-6 secretion and the inhibitory effects of TNF-α in response to PRRSV infection were not different among routes and genotypes (*p>0.05*).

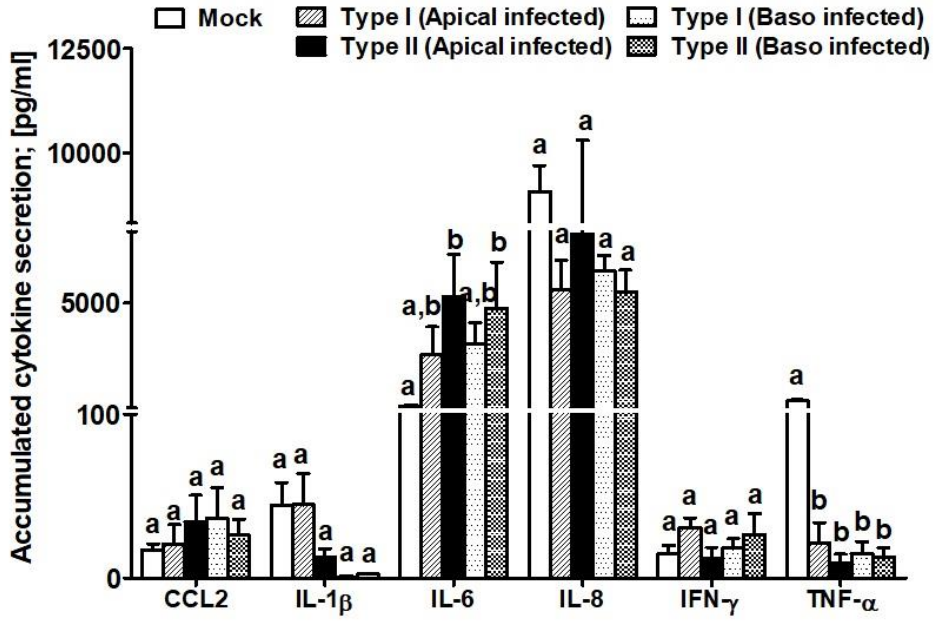


Figure 15 Effects of PRRSV infection on cytokine secretion accumulated at the apical and basolateral compartment of PE cells. The microporous membrane-grown PE cells were infected via apical or basolateral route with mock, PRRSV type I or type II for 1 hr. Sample media from each compartment of PE cells were collected every 2 days for evaluating the amounts of cytokines CCL2, IL-1β, IL-6, IL-8, IFN-γ and TNF-α secretion in response to PRRSV infection for 6 days using enzyme-linked immunosorbent assay (ELISA). Bar graphs represent mean ± SEM in pg/ml (n=5 pigs) of the accumulated concentration of cytokines secreted to media during 0-6 dpi. Bar graph with different letters (a, b) indicates significant difference at $p<0.05$ by one-way ANOVA followed by Newman-Kuel post-hoc test.

PART III: Susceptibility of PRRSV re-infection in the PE cells primary infected with PRRSV regarding to the cellular responses and the mucosal innate immunological system responses

Determination of cytopathic effects and morphologic changes of PE cells in response to PRRSV re-infection

The recent study attempted to examine whether the primary infection of PRRSV modulated PRRSV receptor expression may increase the susceptibility of PE cells to subsequent PRRSV re-infection. Herein, the occurrence of CPE by PRRSV re-infection were observed at 4 dpi (0 day of re-infection), 6 dpi (2 days of re-infection) and 8 dpi (4 days of re-infection) compared to the primary infection. Like the primary infection as shown in the part I, prior to re-infection at 4 dpi, PRRSV mostly caused microscopic changes in the form of syncytial formation and focal degeneration during 4-8 dpi.

At 4 dpi, basolateral with PRRSV produced CPE in all PE cells (100%) (Fig. 16; $p<0.05$). Afterward, at 6 dpi, the CPE induced by PRRSV type II apical infection was raised up to 60% (Fig. 16; $p<0.05$). However, at 8 dpi the area of CPE presented in mock or mock re-infected cells was identical to primary PE cell-infected cells (Fig. 16; $p>0.05$) but could not be identical to those of basolateral type II infected cells (Fig. 16; $p<0.05$).

Re-infected PE with PRRSV type I to the apical side generated the CPE at the same degree as type II re-infected group during 4-6 dpi (Fig. 16; $p>0.05$). CPE at 8 dpi was mostly seen in basolateral type II primary infected PE cells, or basolateral type II re-infected or apical type I- infected PE cells ($p<0.05$). In addition, all supernatant collected from primary infected or re-infected PE cells at 8 dpi revealed CPE area and PRRSV-immunoreactivity at the percentage of 100 per field in MARC-145.

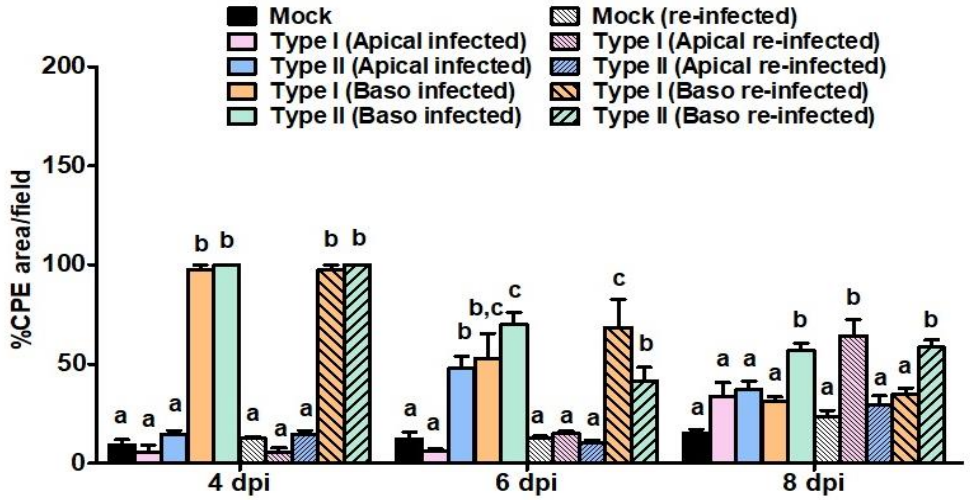


Figure 16 Effects of PRRSV re-infection compared to primary infection on producing cytopathic effects (CPE) in PE cells. The microporous membrane-grown PE cells were infected via apical or basolateral route only at 0 dpi or re-infected with mock, PRRSV type I or type II at 4 dpi for 1 hr. The area of CPE was observed by light microscope and measured at 4, 6 or 8 dpi. Bar graph represents mean ± SEM of % CPE area per field (n=5 pigs). Bar graph with different letters (a, b) indicates significantly different at *p value <0.05* by two-way ANOVA followed by Bonferroni post-hoc test.

Determination of the cellular PRRSV following the PRRSV re-infection in PE cells

Likely, the cellular expression of PRRSV-GP5 by PE cells was observed following the 0-4 day of post-re-infection (= 4-8 dpi). None of the mock infected/re-infected PE cells had PRRSV-GP5 positive cell throughout the experiment (Fig. 17).

Expression of PRRSV-GP5 positive cells had a similar pattern to the CPE effect; gradually increasing by apical PRRSV infection but gradually decreasing by basolateral infection. In apically infected PE cells, PRRSV-GP5 positive cells were higher by type II than type I infection at 4 dpi (Fig. 17; $p<0.05$) (Fig. 17). During 6 dpi, apical type II pre-infection produced PRRSV-GP5 positive cells higher than other apical PRRSV infected groups ($p<0.05$).

Focusing on the re-infection experiments, PPRSV re-infection at the same side as primary infection could not reveal the different numbers of PRRSV-GP5 positive cells from primary infection at 4 dpi (Fig. 17; $p>0.05$). But re-infection with type I at the basolateral side significantly increased positive cells at a higher level than primary infection (Fig. 17: $p<0.001$; compared to mock). In addition, re-infection with type I at the apical side, some PRRSV-GP5 positive cells were found during 4-6 dpi, although there were no differences from primary infection (Fig. 17).

At 8 dpi, all PRRSV infection produced PRRSV-positive PE cells over 30% (Fig. 17; $p<0.05$; compared to mock). Basolateral type I infection and apical type I re-infection significantly demonstrated PRRSV-GP5 positive cells higher than other infected groups ($p<0.05$).

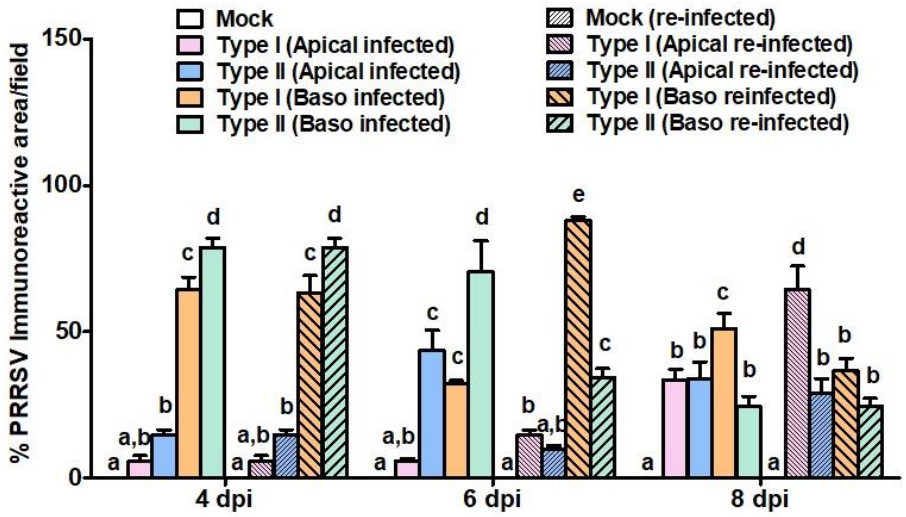


Figure 17 Cellular expression of PRRSV-GP5 protein in response to PRRSV re-infection compared to primary infection in PE cells. The microporous membrane-grown PE cells were infected via apical or basolateral route only at 0 dpi or re-infected with mock, PRRSV type I or type II at 4 dpi for 1 hr. PRRSV protein were evaluated by immunohistochemistry using antibody against PRRSV-GP5 and observed under a light microscope at 4, 6 or 8 dpi. Bar graph represents mean ± SEM of % immunoreactive area per field (n=5 pigs). Bar graph with different letters (a, b, c, d, e) indicates significantly different at $p<0.05$ by two-way ANOVA followed by Bonferroni post-hoc test

Effects of PRRSV re-infection on the TLRs1-10 expression and related cytokine gene expression in PE cells

Figure 18 demonstrated the mRNA expression of TLRs1-10 genes that were observed at 8 dpi (4-day post-re-infection). There was no difference in gene expression between mock and mock re-infection (*p>0.05*; data not shown). Both PRRSV type I and type II infection were found to down-regulate some TLR gene expression, particularly of *TLR5* and *TLR8* (Fig. 18; *p<0.05*). Additionally, *TLR1* expression was down-regulated by type I infection, likely to relate with result at 4 dpi (Fig. 18; *p<0.05*).

Following 4 days of re-infection (at 8 dpi), *TLR1* and *TLR7* were turned up-regulated by type I PRRSV (Fig. 18; *p<0.05*; infected vs. re-infected). Likewise, type II re-infection also increased *TLR1* and *TLR2* expression (Fig. 18; *p<0.05*; infected vs. re-infected).

Changes in expression of interested cytokine genes were also observed at 8 dpi (Fig. 19). Consistent with result from Part II (at 4 dpi), the expression of all related cytokine genes IL-6, IL-8, IFN-γ and TNF-α were not affected by PRRSV primary infection (*p>0.05*). Re-infection with PRRSV type I or type I completely down-regulated *IL-6* expression (*p<0.05*) but not *IL-8, IFN-*γ and *TNF-*α (*p>0.05*). Although the expression of *IFN-*γ was not respond to primary PRRSV infection, re-infection with type I had lower expression of *IFN-*γ than those of type II re-infection (Fig. 19; *p>0.05*; type I re-infected vs. type II re-infected).

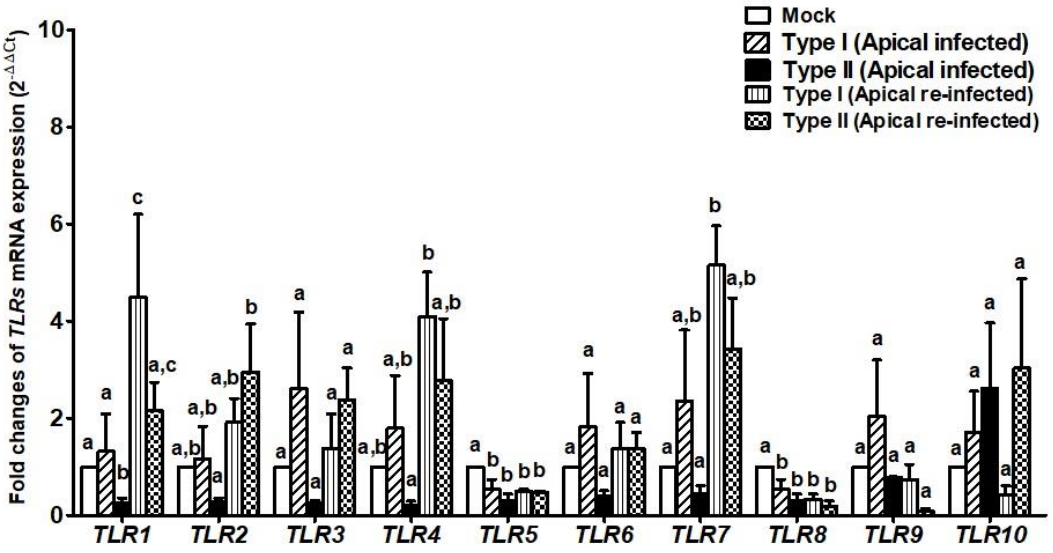


Figure 18 Effects of PRRSV re-infection compared to primary infection on expression of TLRs1-10 in PE cells. PE cells cultured in T25 flask were infected for 1 hr only at 0 dpi or re-infected with mock, PRRSV type I or type II at 4 dpi. Total RNA was isolated at 8 dpi for determining *TLRs1-10* normalized to house-keeping gene *GAPDH* by qPCR. Bar graphs represent mean ± SEM (n=5 pigs) of the fold changes of TLRs mRNA expression from mock using the $2^{-\Delta\Delta Ct}$. Bar graph with different letters (a, b, c) indicates significant difference at $p<0.05$ by one-way ANOVA followed by Newman-Kuel post-hoc test.

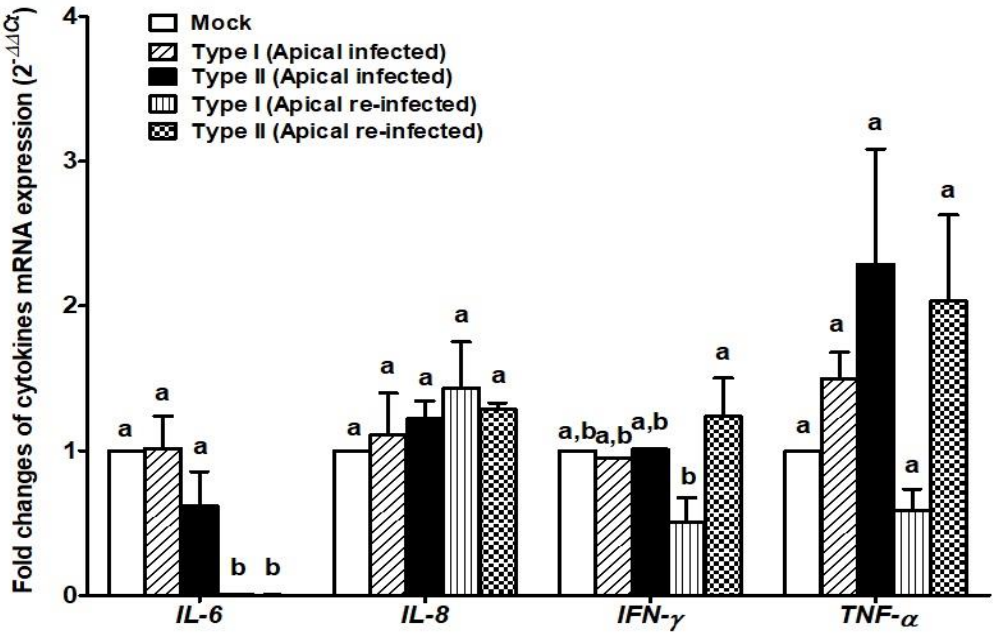


Figure 19 Effects of PRRSV re-infection on mRNA expression of cytokines IL-6, IL-8, IFN-γ and TNF-α expression in PE cells. PE cells cultured in T25 flask were infected for 1 hr only at 0 dpi or re-infected with mock, PRRSV type I or type II at 4 dpi. Total RNA was isolated at 4 dpi for determining *IL-6, IL-8, IFN-γ* and *TNF-α* normalized to house-keeping gene *GAPDH* by qPCR. Bar graphs represent mean ± SEM of the fold changes of cytokine mRNA expression from mock using the $2^{-\Delta\Delta Ct}$ (n=5 pigs). Bar graph with different letters (a, b, c) indicates significantly different at $p<0.05$ by one-way ANOVA followed by Newman-Kuel post-hoc test.

Effect of PRRSV re-infection on the accumulated cytokine secretion in PE cells

In re-infection experiment, PRRSV infection modulating the cytokine secretion was calculated from the accumulation of cytokine concentration to the cell medium compartment during 4-8 dpi. Apical secretion and basolateral secretion of IL-1β, IL-6, IL-8, CCL2, IFN-γ and TNF-α collected every 2 days were demonstrated equally (preliminary data). Thus, the pool data of accumulated cytokine secretion were presented and used for analysis (Fig. 20). At 8 dpi, accumulated secretion of IL-6, IL-8, CCL2, IFN-γ were not different among experimental groups (*p>0.05*). Primary apical or basolateral PRRSV type I infection stimulated IL-1β secretion, but it decreased TNF-α secretion (Fig. 20; *p<0.05*). In primary PRRSV infection, any sides or genotypes decreased TNF-α secretion accumulated during 4-8 dpi (Fig. 20; *p<0.05*). Re-infection with PRRSV seemed to increase IL-1β secretion similar to the primary infection, but not different from mock (Fig. 20; *p>0.05*).

Remarkably, for the abolished TNF-α by PRRSV, it was produced by PRRSV type I apical infection or PRRSV type II basolateral infection, which is in a higher degree than other groups in PE (Fig. 20; *p<0.01*; compared to mock). In addition, re-infection with type II at the apical side of PE (*p<0.01*; compared to mock) lowered the TNF-α secretion higher than primary infection (Fig. 20; *p<0.05*; compared to mock).

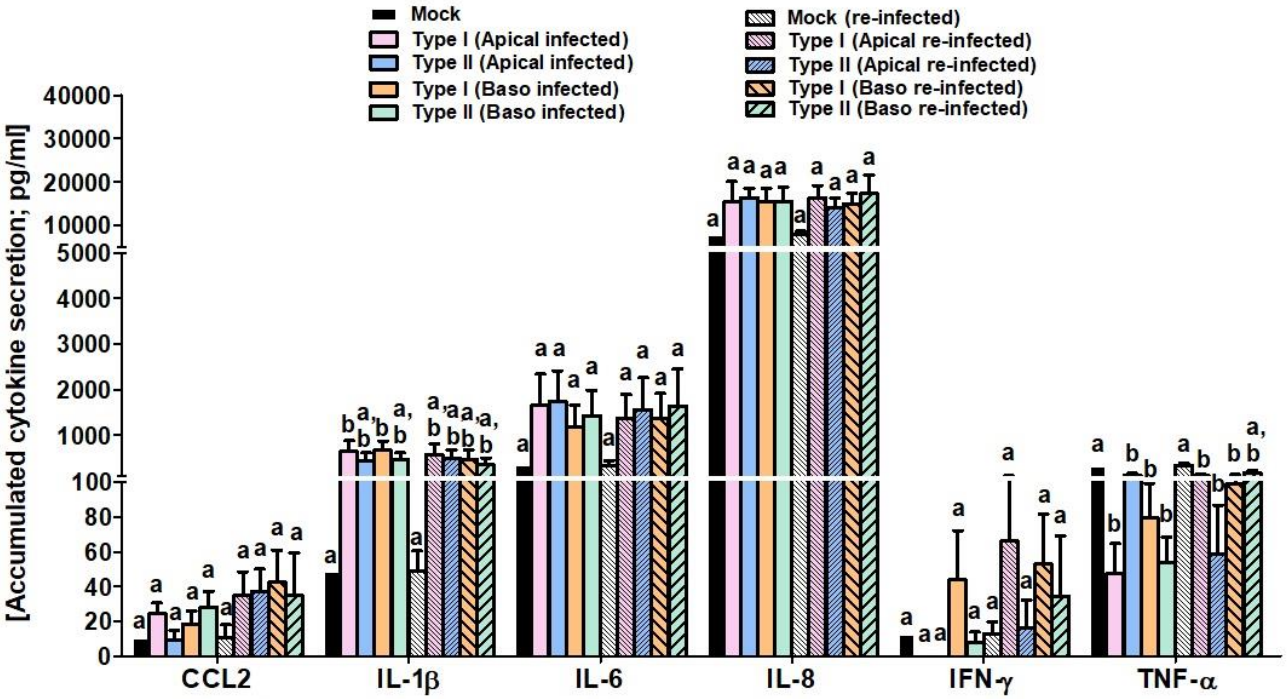


Figure 20 Effects of PRRSV re-infection on cytokine secretion accumulated at the apical and basolateral compartment of PE cells. The microporous membrane-grown PE cells were infected via apical or basolateral route for 1 hr only at 0 dpi or re-infected with mock, PRRSV type I or type II at 8 dpi. Sample media from each compartment of PE cells were collected every 2 days for evaluating the amounts of cytokines CCL2, IL-1β, IL-6, IL-8, IFN-γ and TNF-α secretion in response to PRRSV infection for 6 days using enzyme-linked immunosorbent assay (ELISA). Bar graphs represent mean ± SEM in pg/ml (n=5 pigs) of the accumulated concentration of cytokines secreted to media during 4-8 dpi. Bar graph with different letters (a, b) indicates significantly different at $p<0.05$ by one-way ANOVA followed by Newman-Kuel post-hoc test.

CHAPTER V
DISCUSSION

Infection of PRRSV is limited to some kinds of cells due to the very narrow tropism of PRRSV. Macrophage and monocyte lineages have been reported as the natural target of PRRSV (Duan et al., 1997; Teifke et al., 2001). Expression of PRRSV specific mediators have been identified in PRRSV cell lines, including CL2621, MA-104, and MARC-145 cells (Benfield et al., 1992; Bautista et al., 1993; Kim et al., 1993). Thus, the presence of PRRSV mediators is crucial for determining susceptibility to PRRSV infection by the target cells. Many molecules have been identified as specific mediators, including CD151, CD163, Sn, integrin and vimentin (Zhou and Yang, 2010; Feng et al., 2013). Recently, porcine endometrial endothelial cell line has been generated and examined for PRRSV susceptibility (Feng et al., 2013).

The present study demonstrated that porcine glandular endometrial cells (PE) were additional model that was susceptible to PRRSV. The evidences were supported by firstly the presence of crucial and putative PRRSV mediators, i.e. CD151, Sn, integrin and vimentin on PE. Secondly, virus-host interaction within PE cells was observed following PRRSV infection which consists of changes in microscopic observation, modification of PRRSV mediator gene and protein expression level, and innate immune responses, including TLRs and cytokines. Furthermore, PRRSV-GP5 positive cells were demonstrated and PRRSV from the supernatant of PRRSV infected PE cells were detected. The PRRSV positive in cells and supernatant media of PRRSV-infected cells indicated the occurrence of PRRSV replication and shedding following infection of PE cells. Following PRRSV infection, the occurrence of CPE was related to the percentage of PRRSV-GP5 positive cells. This can be suggested that the virulence of microscopic observation in PE cells is associated with the number of PRRSV. However, the differences between routes of infection or between genotypes of PRRSV in exerting the host response was observed. Some aspects of the PRRSV affecting PE should be discussed and concerned in correlation with the pathogenesis of PRRSV in reproductive failure.

The cellular response of PE cells following PRRSV infection in modification of PRRSV mediators depending on the routes and genotypes

The endometrial epithelial cells lining the uterine cavity can be virally infected from uterine lumen or blood circulation. In the present study, PE cell monolayers were cultured in permeable membrane to compare effects between two sites of infection (apical and basolateral). Basolateral infection simulates the transmission of PRRSV from blood circulation to endometrial cells, whereas apical infection refers to PRRSV transmission from fetus to dam. Our results demonstrated that the apical infection with either PRRSV type I or type II predominately affected PE cells rather than the basolateral infection. The PRRSV-infected cells were supported by a higher degree of CPE, PRRSV-GP5 positive cells. Particularly, PRRSV positive cells were little observed in basolaterally infected PE cells, implying that the persistence of PRRSV in PE cells may be restricted by the route of viral entry. The present study is the first report of PRRSV infection is specific to the apical membrane of polarized endometrial epithelial cell. In the respiratory system, severity and pathogenicity of PRRSV infection result from host-viral interaction at alveolar macrophage but not airway epithelia because the muco-ciliary escalator along the apical surface forms the physicochemical barrier by neutralizing the invading virus (Vareille et al., 2011).

For respiratory syncytial virus (RSV) which favorably infects the apical surface of airway epithelium, it induces the overstated inflammatory responses and mucin secretion which facilitates the colonization of bacteria, allowing cell and fluid accumulation in the lung air spaces (Bousquet et al., 2000). Furthermore, the tight junction protein ZO-1 of the RSV-infected airway epithelia was disrupted, resulting in leaky epithelia (Singh et al., 2007). Therefore, it is speculated that PRRSV-host interaction at the apical aspects of endometrial epithelium might cause the disruption of tight junction barrier leading to fluid accumulation and the consequence of respiratory and reproductive failure.

The PRRSV that transmitted into PE cells were presumably replicated and released into surrounding compartment. This was supported by the findings that MARC-145 cells incubated with culture media collected from apical and/or basolateral compartments of the PRRSV-infected PE cells had positive to PRRSV

proteins correlated with the presence of CPE. All supernatant samples from the infected cells produced PRRSV at the titers of $TCID_{100}$/ ml. Thus, we could not assume that the uptake of PRRSV by PE cells via the apical surface had greater efficiency than the basolateral surface. Perhaps the basolateral membrane presents the structure that impedes PRRSV entry. Alternatively, the replicated PRRSV virions could be equally released from both apical and basolateral surfaces. However, it could be implied from our model using PE cell monolayers that the vertical transmission of PRRSV may occur via the shedding of maternal PRRSV to fetus during pregnancy. Then the *in utero* infected fetus may act as the reservoir of PRRSV for shedding the virus back to their mother. As the apical surface of endometrial epithelium is a side favorable of infections, maternal endometrial cell can be re-infected form PRRSV shedding by infected fetus. This re-circulation of PRRSV between dams and fetus might play the critical role for infected dam as being PRRSV reservoir with a little degree of inflammation or lesion at the placental membrane. Our consideration is supported by the experiment showing that nasal inoculation of PRRSV to pregnant gilts induced high viral load in individual litters which were closely related to the proportion of viral load at the maternal-fetal interface (Ladinig et al., 2015).

Infection with different PRRSV genotypes demonstrated the different severity and clinical outcome (Nelsen et al., 1999). PRRSV type II infection expressed more severe respiratory distress than type I (Nielsen et al., 2002); however, both genotypes cause the reproductive failure at the same degree (Scortti et al., 2006). Our current results showed that cytopathic effect and PRRSV positive of PE cells produced by type II were greater than those by type I. However, in the natural infection or *in vivo* inoculation with PRRSV type II could not demonstrate the virulence of reproductive signs, i.e. viral load in fetus or maternal-fetal interface, numbers of embryonic death or PRRSV-positive litters, that differ from type I (Ladinig et al., 2015). To date, the identified virulent factor among each genotype of PRRSV is limited. Most non-structural glycoproteins (Nsp 3-8) and ORF5, which encode structural GP5, have been identified as the virulence determinant of PRRSV type II (Kwon et al., 2008). Among different PRRSV genotypes, the genetic identity ranges from 55% to 63% for non-

structural proteins (Allende et al., 1999) and from 61% to 81% for structural proteins (Meng et al., 1995; Nelsen et al., 1999). Therefore, the variation of these viral proteins appears to be the determinant of distinct virulence between type I and type II infection.

In the PE cells, we currently showed efficient replication with CPE and viral antigen expression following the direct inoculation implying that PRRSV becomes adapted to other cells, such as endometrial cells other than PAMs. The viral susceptible to host cells will be considered when the viral pathogens interact with host cells, leading to pathogenesis, such as cytocidal infections (Albrecht et al., 1996). The cytocidal infections were associated with the viral-host interaction to support the viral replication and spreading, i.e. modulatory effects on DNA, RNA and protein expression. However, these alterations required the specific viral mediators and machinery produced by host cells.

As aforementioned in PAMs, CD163 was suggested to be a core PRRSV receptor which was facilitated by Sn (Vanderheijden et al., 2003). In our study, a common phenotype of non-infected PE cells was characterized as $Sn^{+}/CD163^{-}$ due to a very low level of CD163; however, these cells could be susceptible to PRRSV. Perhaps, other mediators could representatively play a role in PRRSV disassembly during the early stage of infection due to the shortage of CD163. Other putative PRRSV mediators like CD151, integrin and vimentin were also demonstrated in the non-infected PE cells. However, integrin and vimentin do not serve as specific receptors to PRRSV infection.

Among the putative PRRSV mediators, CD151 is most likely expressed by our PE cell model. Thus, despite the lack of CD163, CD151 may interact with 3'UTR of PRRSV RNA to render the PE cells susceptible to PPRSV infection and viral replication as previously indicated in CD151-transfected MARC-145 cells. In porcine endometrial endothelial cells (PEE), CD151 and Sn were significant molecules for mediating PRRSV infection (Shanmukhappa et al., 2007; Feng et al., 2013). More likely, only the expression of those two mediators, Sn and CD151, is efficient for the porcine endometrium susceptible to primary PRRSV infection. However, PE cells significantly

expressed CD163 following PRRSV infection. This upregulation of CD163 by PRRSV infected PE cells might be mediated through the activation of TLR signaling cascade.

CD151 has been commonly expressed in PRRVS permissive cell lines and also reported in normal and cancer cell lines of human endometrium (RL95-2 and HEC-1-A) (Dominguez et al., 2010). Our study was the first to report the expression of *CD151* in normal porcine endometrial epithelial cells. During 4-6 dpi, the expression of *CD151* mRNA correlated with its target protein was up-regulated by PRRSV type I via the basolateral but not the apical surface of PE cells. This finding prompts us to the surveillance of PRRSV viremia that may establish the endometrium as a site of PRRSV replication. On the other hand, the increased cellular CD151 protein with no significant increase in *CD151* expression was observed following apical PRRSV type II infection. This observation further suggests that apart from viremia, the increased CD151 may play a potential role in PRRSV type II contamination from PRRSV-positive semen or fetus to trigger the endometrium for subsequent PRRSV infection.

Moreover, the up-regulation of *CD151, CD163, Sn* and *integrin* by type I infection was greater than type II. It may be postulated that initial infection with type I could likely promote subsequent infection through type I-induced up-regulated PRRSV mediators. Nevertheless, the Sn was markedly decreased in apically and basolaterally type I-infected cells, suggesting the advantage of type I contamination to decrease the PRRSV entry to the endometrium. Inappropriately, the increased Sn by basolateral type I infection representing the viremia subsequently relapsed at 4 dpi and so did type II infection at the same side. However, the modulation of Sn expression by PRRSV pre-infection on enhancing the subsequent PRRSV infection of porcine endometrium remains to be investigated.

In the current study, apically or basolaterally type I-infected cells were found to decrease both integrin and vimentin expression as compared to type II-infected cells. The down-regulated integrin and vimentin by type I were consistent with its cytopathic effects which were observed in type I less than type II infection. In contrast, the increased integrin in PE cells in response to apical type II infection at 2-6 dpi with the marked presence of CPE including syncytial formation or plaques, and PRRSV-positive cell were the obvious results in this study.

In general, integrin functions to regulate growth and proliferation through G1 phase cyclin dependent cyclase by binding to extracellular matrix (Schwartz and Assoian, 2001). Up-regulation of integrin particularly in apically PRRSV-infected PE cells seem to rely on the survival strategy of viruses that promote cell proliferation via integrin-mediated regulation to increase the population of PRRSV-carried host cells directly. Modification of endometrial integrin by PRRSV is conceivably related to PRRSV-induced reproductive failure and enhances the permissiveness for following PRRSV infection.

In PRRSV type I-infected PE cells, the presence of CPE area was maintained or decreased at 6 dpi whereas the mock-infected PE cells presented the focal degeneration as equally as the PRRSV-infected cells. The degeneration of the non-infected PE cells observed at 6 dpi may be a result of age-associated changes which are characteristics of primary cell culture system but not by the viral effect (Phipps et al., 2007). Nevertheless, the PRRSV-infected cells had less cell degeneration than non-infected PE cells due to the interaction of PRRSV with host to produce cell replacement by stimulating proliferation. The increased integrin expression by PRRSV may associate with PE proliferation leading to the propagation and persistence of virus within host cells as discussed earlier.

In addition, vimentin has been suggested to interact with other cytoskeletal molecules for facilitating PRRSV infection (Huang et al., 2009). In PE cells, the interaction between vimentin and other cytoskeletal filaments possibly occurs and results in the modification of cellular structure and integrity in endometrial cells, particularly the occurrence of syncytial formation. However, the significance of down-regulation of integrin and vimentin by type I and type II infection, which was obviously seen at 6 dpi associated with morphological and physiological changes of PE cells, has to be elucidated.

The infectious cycle of PRRSV is a multistep process that requires several interactions of PRRSV proteins and cellular molecules. The mechanism of PRRSV infection is mediated via specific cellular mediators to permit viral attachment, internalization and uncoating. Sn and CD163 are important mediators that play a potential role for PRRSV internalization and uncoating. Even though the PE cells

characterized as $Sn^{+}/CD163^{-}$ which could not be PRSSV tropism, they could be transformed to $Sn^{+}/CD163^{+}$ and susceptible to the subsequent infection.

Changes in expression of CD163 and Sn may be likely mediated by many cytokines released from PE cells in response to PRRSV through toll-like receptors (TLR)-signaling pathway. Several TLRs can recognize viral nucleic acids or viral protein produced by PRRSV leading to release of many cytokines, such as IL-6, TNF-α and IFN-γ at the early stage of infection (Yoo et al., 2010).

Innate immunological response of PE cells following PRRSV infection

To detect the pathogen molecule, expression and activation of pathogen recognition receptors, particularly TLRs, was frequently demonstrated by other tissues. Molecule of PRRSV can be recognized by TLRs as aforementioned in review literature. Therefore, our study investigated whether TLRs mediated PRRSV infection in endometrial cell. If TLRs mediated PRRSV, changes in innate immunity, i.e. secretion of pro-inflammatory and antiviral cytokines, should be responded.

In the present study, PE monolayer cells revealed low expressions of all TLRs mRNA compared to *GAPDH*. However, TLR2, TLR3, TLR4, TLR5 and TLR7 proteins have been detected in PE cell monolayer (Deachapunya et al., 2012). Moreover, PE cells constitutively secreted IL-1β, IL-6, IL-8 and TNF-α which were mediated by human TLR 1-10 ligands, including poly I:C dsRNA simulating viral nucleic acids (Deachapunya et al., 2012). The secretion of CCL2 and anti-viral cytokines IFN-γ were firstly demonstrated in this study. However, the absence of IL-10 mRNA expression and secretion by PE cells was demonstrated in both mock-treated and PRRSV-infected conditions (data not shown). Thus, PE cells have ability to interact with the viral pathogens to establish a major part of the innate immune system, TLR signaling system and related cytokine synthesis and release. The modulatory effects of primary PRRSV infection on the TLRs system may facilitate both host and pathogen interaction and responding to each other either by neutralizing the invading virus or facilitating viral replication and spreading.

Modulation of TLRs expression by PRRSV infection have been demonstrated elsewhere. However, the results were varied depending on the genotype of PRRSV and the target cells. Pigs infected with PRRSV type II tended to up-regulate the mRNA expression of TLRs 2, 3, 4, 7 and 8 in the lymphoid tissues, which increased susceptibility to secondary pathogens and consequence of severe clinical outcomes (Liu et al., 2009). The highly pathogenic PRRSV, the sub-genotype of type II, was a strong inducer of TLRs 3, 7, and 8 in PAM (Zhang et al., 2013). The expressions of TLR3 in PAM were differentially regulated by the different genotypes of PRRSV (Kuzemtseva et al., 2014).

Infection with different genotypes of PRRSV resulted in differential TLRs expression. The modulated TLRs expression following PRRSV infection may reflect the susceptibility or resistance of the cell for secondary infection. In PE cells, up-regulation of *TLR1* and *TLR3* was produced by apical type I infection. As TLR3 functions to recognize the dsRNA virus, TLR3 activation initiated by PRRSV type I infection in PAM resulted in increased TLR3 and IFN-β to suppress PRRSV infectivity (Sang et al., 2008). In the present study, IFN-β was not detected in PE (preliminary data) which was consistent with less CPE and PRRSV positive cells infection by type I reflecting its low infectivity being observed following type I infection. On the other hand, *TLR10* highly expressed in B cells and weakly expressed in plasmacytoid dendritic cells (PDC) and endometrium (Hornung et al., 2002). To our knowledge, TLR10 may be essential for the adaptive immunity, but not restricted to the endometrium epithelia.

Due to the function of TLR4, which recognizes the lipopolysaccharides of gram-negative bacteria (Takeda and Akira, 2004), down-regulation of TLR4 mRNA expression by both genotypes of PRRSV may reduce the ability to neutralize the effect of secondary infection by gram negative bacteria.

Although the up-regulated expression of TLRs might increase the protective activity of the cell against the infection. It is possible that the protective response might be exacerbated, causing harmful to the cells, such as excessive promoting the pro-inflammatory cytokines. During pregnancy, the T helper 1/ T helper 2 cytokine

balance is shifting to a predominance of T helper 2 cytokines to create the tolerance to the fetal allograft (Chaouat, 2007). Thus, the excessive production of T helper 1 pro-inflammatory mediated via TLRs may cause the negative effect to pregnancy resulting in pregnancy failure.

As discussed earlier, TLRs are necessary for immune response against viral infection. In particular, the membrane TLRs, TLR2 and TLR4, recognize the viral proteins, whereas the cytoplasmic TLRs, TLR3, TLR7, TLR8 and TLR9, detect the viral nucleic acids, DNA or RNA virus. It is possible that PRRSV nucleic acids or their proteins may be recognized by TLRs instead of PRRSV mediators. Ligand binding to TLR transduces intracellular signal through NF-κB-dependent or -independent pathways, leading to the transcription of cytokines and chemokines (Akira, 2006).

Without PRRSV infection, IL-8, IL-6 and TNF-α were constitutively produced while low levels of CCL2 and IFN-γ were detected in the PE cells. All PRRSV type II infection stimulated IL-6 secretion whereas no changes in CCL2, IL-8, IL-1β or IFN-γ secretion were detected in response to any PRRSV infection. IL-6 has several effects on immune cells, including recruiting neutrophils to sites of infection at the early stage of infection (Ataie-Kachoie et al., 2014) and switching the pattern of immune cell infiltration from neutrophil to monocytes at later infection stage (Hurst et al., 2001). In addition, increased IL-6 secretion by PRRSV-infected pigs associated to virulent clinical signs by PRRSV type II, contributed to the severity of lung disease in pigs (Renukaradhya et al., 2010). Thus, up-regulation of IL-6 secretion by PRRSV type II but not type I indicates the virulent of this genotype to PE cells and might associated to the intensive morphological changes of PRRSV type II-infected PE cells.

Two important chemokines observed in this study are IL-8 and CCL2. Release of IL-8 by the female reproductive tissue functions to recruit the leukocytes to the endometrium and is so called the first line defense (Arici et al., 1998). Instead, the level of IL-8 in infected cells seems to be maintained as seen in lymphoid tissues collected from PRRSV-persistent pigs (Lunney et al., 2010). Although up-regulation of serum IL-8 and IL-1β were indicated early after PRRSV infection, the up-regulation of three innate markers, IL-8, IL-1β and IFN-γ, were not correlated with the viral level in

PRRSV persistent pigs (Lunney et al., 2010). Another chemokine, CCL2, induces the migration of monocytes from blood to become tissue macrophages and displays chemoattractic activity for memory T cells, natural killer (NK) cells, and perhaps dendritic cells to recruit these cells to sites of tissue injury and stimulate inflammatory responses (Balkwill, 2004). Increase CCL2, IFN-α, and IFN-γ level in gilt serum by PRRSV type II correlated to PRRSV replication in fetal tissue and numbers of fetal death. Thus up-regulation of three cytokines by PRRSV plays an important role for the reproductive effects of PRRSV (Ladinig et al., 2014; Ladinig et al., 2015). However, the secretion of CCL2 by PRRSV infected PE cells was low and did not significant to mock infected cells. This is indicated that when the endometrium was infected with PRRSV, the migration of monocytes and macrophages to the site of endometrial infection might be inhibited and resulted in persistent infection through the suppression of CCL2.

Moreover, the level of up-regulated TLR3 by PRRSV leading to the induction of TNF-α synthesis was apparently relevant to the replication levels of PRRSV (Kuzemtseva et al., 2014). Both *in vivo* and *in vitro* studies suggested that PRRSV up-regulated *TNF-α* expression of infected cells and resulted in induction of apoptosis in uninfected bystander cells in the lungs of pigs (Choi and Chae, 2002). On the contrary, TNF-α production and secretion was reduced in PRRSV-infected PAMs (Lopez-Fuertes et al., 2000). These results were consistent with our finding showing that PRRSV infection down-regulated the secretion of TNF-α by PE cells. Since the PRRSV-immunoreactivity and CPE were found in infected PE, it is suggested that the replication and cytocidal effects of PRRSV directly on endometrium may be mediated by the decreased TNF-α with no-upregulated IL-8, and IFN-γ.

In fact, incubation with recombinant TNF-α was reported to reduce viral replication of PRRSV (Lopez-Fuertes et al., 2000). Alternatively, suppression of TNF-α may be one of evasion strategies of PRRSV to preserve their host cells for replication and to prevent the elimination that would be the outcome from activation of TNF-α. In the study of CPE (Fig. 9), the degenerated cells in mock group at 6 dpi were higher than infected PE group implying that the preservation of PE cells induced by PRRSV,

was relevant to the decreased TNF-α (Fig. 15). The suppression of TNF-α by PRRSV was carried out by Nsp1. Both Nsp1α and Nsp1β were demonstrated to suppress transcription of TNF-α, by inhibiting the activation of NF-κB and sp1 on promotor region of TNF-α (Subramaniam et al., 2010)

Additionally, PAMs and PBMC from pigs infected with PRRSV either type I or type II in were reported to induce immunosuppressive condition via the up-regulation of IL-10 (Suradhat and Thanawongnuwech, 2003). However, the cytokine secretions of IL-10 and IFN-α by PE cells were not changed following PRRSV infection (data not shown).

Together, PE cells have been demonstrated the mucosal innate immune response against PRRSV by up-regulating *TLRs 1, 3* and *10* expression and increasing cytokine secretion of IL-6. Meanwhile, the suppression of innate immune was also demonstrated by the down-regulating *TLR4* expression and suppression of many cytokines, particularly TNF-α. Therefore, the total response was not sufficient to eliminate PRRSV infection. PRRSV infected endometrium can play an important role in the site of reservoir that results in persistent infection.

Susceptibility of PRRSV re-infection in the PE cells pre-infected with PRRSV

During infection, PRRSV can be harvested and maintained in macrophages, the natural target of PRRSV. In infected macrophage, many immunological modulations are produced by PRRSV, i.e. changes of TLRs expression, modulation of cytokines and IFN production. These modulatory effects of pre-infection correlated with the increased viral replication in cell tropism. In addition, adaptive immune response is often weak and delayed. More likely, the immunomodulatory property of PRRSV facilitates the characteristic of persistent infection. Certainly, PRRSV re-infection can be occurred due to the re-circulation of shedding virus. Therefore, understanding host immune responses following re-infection with PRRSV could help to develop the effective prevention strategies for PRRSV such as more efficacious vaccines.

From the study of primary PRRSV infection in part I, the occurrence of CPE and PRRSV positive cells by apical infection seems to be more virulent than

basolateral infection at 2 dpi, but gradually decreased from 4 dpi to 6 dpi. However, in re-infection experiment (part III) which performed separately from part I and II, the infectivity of primary PRRSV at the basolateral side of PE was studied at 4 dpi and revealed higher than others. However, at 6 dpi to 8 dpi, cellular effects and PRRSV-positive cells via apical or basolateral infection were not different. Although infection with PRRSV type I demonstrated virulence less than type II during 0-6 dpi, CPE and PRRSV-positive cells were increased and maintained at the same degree to infection with type II at 8 dpi. Interestingly, type I apically re-infected cells predominantly presented CPE and PRRSV-positive cells at 8 dpi. Therefore, infection with type I causes persistent infection via inducing slow onset. Moreover, PRRSV type I can turn virulent subsequently re-infection with homologous genotype.

The different result between 2 experiments (part I and part III) indicated time-dependent effect of PRRSV virulence that could be explained as followed; 1) during early stage, apical infection is a preferred side of infection; 2) the reduction of PRRSV by primary basolateral infection may be due to the release of all PRRSV new virions to environment; 3) at the later stage, the progeny of PRRSV secreted by the infected PE cells might be freely distributed across the 2 compartments and resulted in the equilibrium of virus. Noticeably, CPE, PRRSV-positive cells and supernatant produced by only primary infection or re-infection were continuing over the time of observation suggesting the persistence of PRRSV in PE has been prompted and re-circulated at the primary infection.

In addition, innate immune response mediated by TLR signaling system and related cytokine synthesis and release was affected by primary PRRSV infection and quietly modulated by PRRSV re-infection (Fig. 18 and 19). As shown in previous studies in part II, primary infection with PRRSV for 4 days, TLR1 and TLR3 were increased by type I, and TLR10 mRNA were increased by type II whereas TLR4 mRNA were decreased by both genotypes. In contrast, when primary infection with PRRSV observed for a longer period (8 dpi), none of TLRs mRNA was induced by PRRSV infection. However, *TLR1* was decreased by type II and *TLR5* and *TLR8* were decreased by both genotypes. Re-infection with type I turned to enhance the expression of *TLR1*, although it could not modulate the suppression effects of

primary infection. Up-regulation of *TLR1* by type I was consistent between response of infection and re-infection at 4 days (4 dpi and 8 dpi, respectively), indicating that *TLR1* served as early target gene of PRRSV type I. Down-regulation of *IL-6* expression by PRRSV re-infection (type I and type II) and *TLR5* and *TL8* by PRRSV seems that the suppression of innate immunity by PRRSV in endometrium is developed over the time of PRRSV presented in host at the first contamination, whether or not presence of re-infection. It is possible that PE can provide viral mediators and innate immunity, i.e. TLRs system, pro-inflammatory and anti-viral cytokines, to protect itself. But PE-viral interaction seems to compromise with PRRSV and allow PRRSV to modulate in order to survive and re-circulate for a lifetime.

Production of IL-1 β secretion by HP-PRRSV infected PAMs was reported through the activation of NF-κB, ERK1/2, and p38 MAPKs, resulting in virulent by this genotype (Bi et al., 2014). Consistently, increase IL-1β secretion by PRRSV, particularly by PRRSV type I apical/basolateral infection at 8 dpi indicated that PRRSV type I turned virulent at later stage of infection. Induction of leaky epithelia was demonstrated by incubation of Caco2 (human epithelial colorectal adenocarcinoma) cells with IL-1β (Al-Sadi and Ma, 2007) . Moreover, permeability of PE cells was increased following PRRSV infection (unpublished data by our lab). Perhaps PRRSV induced IL-1β by PE cells was relevant to disruption of endometrial integrity and might be the cause of reproductive failure.

Few *in vivo* studies regarding re-infection of PRRSV have been demonstrated. Shibata et al. (2000) inoculated PRRSV to pigs and re-inoculated with the homologous PRRSV genotypes at 77-day post inoculation, showing that PRRSV clinical signs were improved and the viremia was reduced after second exposure. However, the previous exposure prevents only the severity of infection, but could not eliminate the infection. In addition, the absolute protective effect of the consecutive homologous challenge of the previous infection was demonstrated by Weesendorp et al. (2016). They demonstrated that after re-infection, serum form PRRSV infected pig had homologous neutralization, up-regulation of PRRSV-neutralizing antibody titer and increased IFN-γ producing cells. It was suggested that the mechanism of PRRSV

clearance following the second challenge might relate to the activation of adaptive immunity, in particular the activation of the memory response (Weesendorp et al., 2016).

However, the observation of host immune response by our model using primary porcine endometrial cells is limited to the innate immunity. Indeed, the activation of immunological response by PRRSV previous infection requires the activation of both innate and adaptive responses in developing the sufficient protection against secondary exposure. Co-culture system between PE cells and immune cells should be performed for further investigation. Additionally, it would be of interest to investigate the effect of heterologous challenges in further study.

CHAPTER VI
CONCLUSION

This research demonstrated the susceptibility of porcine endometrial cells (PE) to direct PRRSV infection. The possibility of PE cell permissiveness to PRRSV was demonstrated in which many putative PRRSV mediator proteins were expressed. The cytopathic effects and PRRSV-immunoreactive area in infected PE produced by PRRSV were determined as the susceptibility or infectivity. PRRSV-host interactions were observed as cellular and immune effects, including modulation of PRRSV mediators, *TLR* gene expression and related cytokine secretion, by PRRSV infection. Infection with different PRRSV genotypes and different routes resulted in different outcomes. Apical infection, particularly PRRSV type II, produced more CPE and PRRSV positive cell than others. By contrast, infection by PRRSV type I up-regulated PRRSV mediator protein expression at many time points more than type II. In PE cells, PRRSV had genomic effects of TLR genes with distinct pattern depending on genotype. Both genotypes modulated cytokine secretion. Nevertheless, the homologous re-infection of PRRSV did not demonstrate the different pathogenicity from the previous infection.

The main findings can be concluded as follows:

1. PE cells are PRRSV permissive and can be directly infected with PRRSV.
2. Susceptibility of endometrium to PRRSV is different depending on routes and courses of infection. At early stage, PRRSV has preferential infection to apical membrane, whereas basolateral infection is preferred later.
3. Infection with PRRSV type II has more virulent to PE cells than type I but type I infection leads to susceptibility for subsequent infection more than type II.
4. Modification of TLRs expression depends on duration of PRRSV infection. At early infection, *TLR1*, *TLR3*, *TLR10* and *TLR4* expression are the target genes of PRRSV. Later, *TLR1*, *TLR2*, *TLR4*, *TLR5* and *TLR8* expression are affected.

Different genotypes of PRRSV modulate the distinct pattern of each target gene.

5. PRRSV type II increased secretion of IL-6 at early infection. Alternatively, both genotypes of PRRSV dampen the secretion of TNF-α.
6. Changes in the expression and functions of PRRSV mediators, TLRs and cytokines by primary PRRSV infection could be applicable for the evasive strategies for PRRSV persistence and re-circulation in reproductive organs.
7. PRRSV-PE interaction on innate immune system may result in inappropriate innate immune response, which is promoting the susceptibility for subsequent PRRSV infection or complicating infection by other pathogens.

The present study is the first time that demonstrates the different infectivity between PRRSV type I and type II in porcine endometrium. The apical infection acts as the critical route of PRRSV complications in reproductive tissue. PRRSV from contaminated semen or infected fetuses directly infect to apical membrane of endometrium and leads to PRRSV replication and re-circulation. In addition, basolateral infection which is transmitted via blood circulation had less virulent, but its cellular effects may associate with the persistent PRRSV in herds. The recent information may be beneficial for implication of the appropriate strategy of PRRSV prevention.

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