การกระตุ้นการสร้างไซโตคายน์ interleukin-1 receptor antagonist (IL-1Ra) โดย
ไวรัส porcine reproductive and respiratory syndrome virus (PRRSV)

นายธีรวุฒิ เนตรอำพันธ์

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Induction of interleukin-1 receptor antagonist (IL-1Ra) by porcine reproductive and respiratory syndrome virus (PRRSV)

Mr. Teerawut Nedumpun

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By	Mr. Teerawut Nedumpun
Field of Study	Medical Microbiology
Thesis Advisor	Professor Dr. Sanipa Suradhat, D.V.M., Ph.D.
Thesis Co-Advisor	Associate Professor Dr. Tanapat Palaga, Ph.D.
	Assistant Professor Dr. Patcharee Ritprajak, D.D.S., Ph.D.

Accepted by the Graduate School, Chulalongkorn University in Partial Fulfillment of the Requirements for the Doctoral Degree

.....Dean of the Graduate School

(Associate Professor Dr. Thumnoon Nhujak, Ph.D.)

THESIS COMMITTEE

.....Chairman

(Professor Dr. Nattiya Hirankarn, M.D., Ph.D.)

.....Thesis Advisor

(Professor Dr. Sanipa Suradhat, D.V.M., Ph.D.)

.....Thesis Co-Advisor

(Associate Professor Dr. Tanapat Palaga, Ph.D.)

.....Thesis Co-Advisor

(Assistant Professor Dr. Patcharee Ritprajak, D.D.S., Ph.D.)

.....Examiner

(Associate Professor Dr. Chintana Chirathaworn, Ph.D.)

.....Examiner

(Assistant Professor Ekasit Kowitdamrong, M.D.)

.....External Examiner

(Professor Dr. Porntippa Lekcharoensuk, D.V.M., Ph.D.)

ธีรวุฒิ เนตรอำพันธ์ : การกระตุ้นการสร้างไซโตคายน์ interleukin-1 receptor antagonist (IL-1Ra) โดยไวรัส porcine reproductive and respiratory syndrome virus (PRRSV) (Induction of interleukin-1 receptor antagonist (IL-1Ra) by porcine reproductive and respiratory syndrome virus (PRRSV)) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ศ. สพ.ญ. ดร. สันนิภา สุรทัตต์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. ดร. ธนาภัทร ปาลกะ, ผศ. ทพ.ญ. ดร. พัชรี ฤทธิ์ประจักษ์, 130 หน้า.

ไวรัส porcine respiratory and reproductive syndrome virus หรือไวรัส พี อาร์ อาร์ เอส เป็นเชื้อจุลชีพก่อโรคในสุกรซึ่งเป็นปัญหาสำคัญในระบบอุตสาหกรรมการเลี้ยงสุกรทั่วโลก เชื้อไวรัสพีอาร์อาร์เอสมีความสามารถในการกดภูมิคุ้มกันโดยอาศัยกลไกการหลบหลีกภูมิคุ้มกันหลากหลายรูปแบบ ไซโตคายน์ interleukin-1 receptor antagonist หรือ IL-1Ra เป็นไซโตคายน์ชนิดต่อต้านการอักเสบ (inhibitory cytokine) ซึ่งจะถูกสร้างในช่วงต้นของการตอบสนองทางภูมิคุ้มกันโดยทำหน้าที่ควบคุมการทำงานของระบบภูมิคุ้มกันแบบไม่จำเพาะ (innate immunity) และการตอบสนองทางภูมคุ้มกันของเซลล์เม็ดเลือดขาวชนิดลิมโฟไซต์ (lymphocytes) ในการศึกษานี้มีวัตถุประสงค์เพื่อจะตรวจหาการกระตุ้นการสร้างไซโตคายน์ IL-1Ra โดยไวรัส พี อาร์ อาร์ เอส รวมทั้งอิทธิพลของไซโตคายน์ IL-1Ra ที่มีต่อการกดการตอบสนองทางภูมิคุ้มกันของสุกร

การศึกษานี้เริ่มต้นด้วยการพัฒนาระบบการเลี้ยงเซลล์ชนิด monocytes-derived dendritic cells (MoDC) เพื่อใช้สำหรับการศึกษาผลจากการติดเชื้อไวรัส พี อาร์ อาร์ เอส ต่อการทำงานของเซลล์ภูมิคุ้มกันสุกรในห้องปฏิบัติการ โดยผลจากการศึกษาพบว่า การพัฒนาระบบการเลี้ยงเซลล์ชนิด MoDC สามารถลดจำนวนเซลล์ตั้งต้นชนิดโมโนไซต์ (monocytes) เพื่อใช้ในการสร้าง MoDC ให้น้อยลงได้ และยังสามารถเพิ่มจำนวนเซลล์ที่แสดงออก CD1 บนผิวเซลล์ ซึ่งใช้เป็นตัวบ่งชี้คุณสมบัติของเซลล์ MoDC ได้มากขึ้น นอกจากนี้ยังพบว่าเซลล์ MoDC ที่ได้มานั้น มีความสามารถในการเก็บกิน (phagocytosis) และสร้างไซโตคายน์ชนิด IFN-γ มากขึ้นอีกด้วย ดังนั้นจึงสรุปได้ว่าการพัฒนาระบบการเลี้ยง MoDC ทำให้ได้เซลล์ MoDC ที่มีประสิทธิภาพที่ดีขึ้น เหมาะสมที่จะใช้ในการศึกษาในห้องปฏิบัติการต่อไป ในส่วนการศึกษาการกระตุ้นการสร้างไซโตคายน์ IL-1Ra โดยไวรัส พี อาร์ อาร์ เอส พบว่าไวรัส พี อาร์อาร์ เอส ชนิดที่ 2 มีความสามารถในการกระตุ้นการสร้าง IL-1Ra ทั้งในระดับการแสดงออกของยีนส์ และระดับการสร้างโปรตีน โดยให้ผลในรูปแบบเดียวกันทั้งในห้องปฏิบัติการและในสุกรที่ได้รับวัคซีนไวรัสพีอาร์อาร์เอสเชื้อเป็น โดยพบว่าเซลล์ในกลุ่ม myeloid เป็นเซลล์หลักที่สร้างไซโตคายน์ IL-1Ra นอกจากนี้ยังพบว่า เชื้อไวรัส พี อาร์ อาร์ เอส สายพันธ์ก่อโรครุนแรง (highly pathogenic PRRSV) ไม่มีความสามารถในการกระตุ้นการแสดงออกของยีนส์ IL-1Ra ในสุกรที่ได้รับเชื้อ จากนั้นได้ทำการศึกษาอิทธิพลของไซโตคายน์ IL-1Ra ที่มีผลต่อการกดการตอบสนองทางภูมิคุ้มกันของสุกรในห้องปฏิบัติการ โดยใช้วิธี IL-1Ra neutralization พบว่าไซโตคายน์ IL-1Ra ที่สร้างจากการถูกกระตุ้นโดยไวรัส พี อาร์ อาร์ เอส มีฤทธิ์กดการทำหน้าที่ของเซลล์ในระบบภูมิคุ้มกันชนิด innate ได้แก่ การเก็บกิน การแสดงออกของ MHC II และ CD86 บนผิวเซลล์ รวมทั้งการแสดงออกของยีนส์ที่สร้างไซโตคายน์ชนิด IL-1 และ IFN-α รวมทั้งกดการพัฒนา (differentiation) และการเพิ่มจำนวน (proliferation) ของเซลล์เม็ดเลือดขาวชนิดทีลิมโฟไซต์ได้ นอกจากนี้เป็นที่น่าสนใจว่า แม้ว่าการกระตุ้นการสร้างของไซโตคายน์ IL-1Ra โดยไวรัสจะไม่มีผลต่อการเพิ่มขึ้นของไซโตคายน์ IL-10 แต่กลับมีผลต่อการเพิ่มจำนวนของเซลล์ทีลิมโฟไซต์ชนิด regulatory T lymphocytes (Treg)

จากการศึกษานี้สรุปได้ว่า ไวรัส พี อาร์ อาร์ เอส มีความสามารถในการกระตุ้นการสร้างไซโตคายน์ IL-1Ra เพื่อใช้ในการกดการตอบสนองทางภูมิคุ้มกันสุกร ซึ่งสามารถช่วยอธิบายพยาธิกำเนิดของโรค และการเหนี่ยวนำภาวะการกดภูมิคุ้มกันในสุกรที่ติดเชื้อไวรัสพี อาร์ อาร์ เอส ได้อย่างเป็นรูปธรรม นอกจากนี้ยังสามารถใช้เป็นข้อมูลพื้นฐานในการพัฒนาวิธีการป้องกันและควบคุมโรคต่อไปในอนาคตอีกด้วย

สาขาวิชา จุลชีววิทยาทางการแพทย์ ลายมือชื่อนิสิต .....

ปีการศึกษา 2560 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

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TEERAWUT NEDUMPUN: Induction of interleukin-1 receptor antagonist (IL-1Ra) by porcine reproductive and respiratory syndrome virus (PRRSV). ADVISOR: PROF. DR. SANIPA SURADHAT, D.V.M., Ph.D., CO-ADVISOR: ASSOC. PROF. DR. TANAPAT PALAGA, Ph.D., ASST. PROF. DR. PATCHAREE RITPRAJAK, D.D.S., Ph.D., 130 pp.

Porcine respiratory and reproductive syndrome virus (PRRSV) is one of the major pathogens affecting pig production industry worldwide. Impaired innate and adaptive immune responses are evidenced through the course of PRRSV infection. Several evidences indicate that PRRSV suppresses host immune responses via several immune evasion strategies. Interleukin-1 receptor antagonist (IL-1Ra) is known as an early inhibitory cytokine that suppresses innate immune functions and T lymphocyte responses. The aims of this study were to explore the induction of IL-1Ra by PRRSV and the negative immunomodulatory effects of PRRSV-induced IL-1Ra on porcine immune responses.

In this study, the previous monocytes-derived dendritic cells (MoDC) generation protocol was modified, based on the human and mouse primary DC culture system. The modified protocol required fewer monocytes, but generated higher numbers of $CD1^+$ MoDC. The MoDC from the modified protocol also exhibited increased antigen uptake and IFN-γ production. Therefore, the modified protocol is expedient and reliable for generating potent MoDC. The induction of IL-1Ra by PRRSV was determined both *in vitro* and *in vivo*. Type 2 PRRSV increased both *IL1RA* gene expression and IL-1Ra protein production in the cultured porcine leukocytes. The enhanced production of IL-1Ra was further confirmed in the pigs immunized with a modified-live PRRSV vaccine. Myeloid cell population appeared to be the major IL-1Ra producer in the system. In contrast to the type 2 PRRSV, the highly pathogenic (HP) PRRSV did not induce *IL1RA* gene expression. The immunomodulatory roles of type 2 PRRSV-induced IL-1Ra on porcine immune responses were further explored using an *in vitro* IL-1Ra neutralization assay. The findings demonstrated that PRRSV-induced IL-1Ra was responsible for inhibition of phagocytosis, expressions of MHC II (SLA-DR) and CD86 molecules, as well as down regulation of *IFNA* and *IL1* gene expression. Furthermore, IL-1Ra obtained from PRRSV-infected MoDC also interfered with effector T lymphocyte differentiation and proliferation. Interestingly, although PRRSV-induced IL-1Ra was not directly linked to the IL-10 production, it contributed to the differentiation of porcine regulatory T lymphocytes (Treg).

Our findings demonstrated that PRRSV could enhance IL-1Ra production in infected pigs. Moreover, PRRSV-induced IL-1Ra possessed negative immunomodulatory effects on porcine innate immune functions and T lymphocyte responses. The elucidated roles of IL-1Ra from this study help completing the understanding in mechanism of PRRSV immunopathogenesis and may eventually lead to better disease intervention

Field of Study: Medical Microbiology
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Student's Signature .....
Advisor's Signature .....
Co-Advisor's Signature .....
Co-Advisor's Signature .....

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List of abbreviations

APC	antigen presenting cells
APC	allophycocyanin
BIOT	biotin
BSA	bovine serum albumin
bp	base pair
°C	degree Celsius
CD	cluster of differentiation
CMI	cell-mediated immune
CMV	cytomegalovirus
COX	cyclooxygenase
CSFV	classical swine fever virus
CU-VDL	Veterinary Diagnostic Laboratory, Chulalongkorn University
Cy5	cyanine 5
dpi	day post-infection
etc	et cetera
EU	European genotype
g	gram (s)
FITC	fluorescein isothiocyanate
FMO	fluorescent minus one
FOXP3	forkhead box P3
GM-CSF	granulocyte-macrophage colony-stimulating factor
HCV	hepatitis C virus
HP-PRRSV	highly pathogenic-PRRSV
HIV	human immunodeficiency virus
HSV	herpes simplex virus

IFN	interferon
Ig	immunoglobulin
iNOS	nitric oxide synthase
ISG	interferon-stimulated gene
IL	interleukin
IL-1R	interleukin-1 receptor
IL-1Ra	interleukin-1 receptor antagonist
IMDM	Iscove's Modified Dulbecco's Media
IPMA	indirect immunoperoxidase monolayer assay
LPS	lipopolysaccharide
mAb	monoclonal antibody
MAPK	mitogen activated protein kinase
MHC	major histocompatibility complex
ml	mililitre (s)
mM	milimole
µl	microlitre
MoDC	monocyte-derived dendritic cells
m.o.i.	multiplicity of infection
NF-KB	nuclear factor-KB
NK	natural killer cells
N protein	nucleocapsid protein
Nsp	non-structural protein
ORF	open reading frame
ORF7	nucleocapsid of PRRSV
PAM	pulmonary alveolar macrophage
PBL	peripheral blood leukocyte(s)
PBMC	peripheral blood mononuclear cell(s)

PBS	Phosphate buffered saline
PE	phycoerythrin
PHA	phytohaemagglutinin
PRDC	porcine reproductive and respiratory disease complex
PRRSV	porcine reproductive and respiratory syndrome virus
rpm	rounds per minute
RT-PCR	reverse transcription polymerase chain reaction
SIV	swine influenza virus
TCID50/ml	tissue culture infectious dose
TGF	transforming growth factor
TNF	tumor necrosis factor
Treg	regulatory T lymphocyte(s)
US	American genotype
xg	times gravity

CHAPTER I

Introduction

1.1 Importance and rationale

Porcine reproductive and respiratory syndrome virus (PRRSV) is a swine viral pathogen, which causes serious economic losses in pig industry worldwide. It has been well established that PRRSV induces immunosuppression on both innate and adaptive immune responses (Lunney et al., 2016). Following PRRSV infection, infected pigs usually develop secondary complications known as, porcine reproductive and respiratory disease complex (PRDC) (Rossow et al., 1995) or experience vaccination failures (Suradhat et al., 2006). PRRSV primarily infects antigen presenting cells (APC) including macrophages and dendritic cells, which affects antigen processing and presentation (Charerntantanakul et al., 2006; Rodriguez-Gomez et al., 2013). Compare to other swine viruses, PRRSV remarkably induces weak and delayed innate cytokine productions, including IL-1, IL-6, TNF-α and type I IFN during 7 days post infection (dpi) (van Reeth and Nauwynck, 2000). Furthermore, PRRSV also interferes with adaptive immune responses, leading to delayed induction of viral-specific neutralizing antibodies and interferon-γ producing cells (Butler et al., 2014; Mulupuri et al., 2008). The findings suggested that PRRSV infection strongly affects the overall immunocompetency of the infected pigs.

Interleukin-10 (IL-10) or regulatory T lymphocytes (Treg) has broad immuno-inhibitory properties (Vignali et al., 2008). In PRRSV infection model, PRRSV-induced IL-10 production can be detected in lung and peripheral blood mononuclear cells (PBMC) from 3 and 5 dpi, respectively (Chung and Chae, 2003; Suradhat et al., 2003). In addition, increased numbers of circulating Treg can be observed at 10 dpi (Silva-Campa et al., 2012; Wongyanin et al., 2010). According to the evidence of inductions of IL-10

and Treg in PRRSV infected pig, it was believed that induction of IL-10 and Treg are part of viral-induced immune evasion strategies affecting induction of viral-specific adaptive immune responses (Silva-Campa et al., 2009; Suradhat et al., 2003; Wongyanin et al., 2012). However, when considering the timing of PRRSV-induced IL-10 and Treg in the infected pig, the findings could not fully explain the reduction of innate cytokines during the earlier period of infection, which occurred during the first 3 dpi.

Innate immunity plays a key role on modulation of efficient host specific immune responses against invading pathogens. Pro-inflammatory cytokines (IL-1, IL-6 and TNF-α) and antiviral cytokines (type I interferon) are early innate cytokines, which are produced from epithelium, phagocytic cells and APC. Pro-inflammatory cytokines (IL-1, IL-6 and TNF-α) are essential for induction of pathogen-specific immune responses including T-lymphocyte proliferation and recruitment of T-lymphocytes into infected area (Lacy, 2015). Type I interferon (Type I IFN) is critical for blocking of viral replication and early viral elimination (Braciale et al., 2012). Poor induction of innate immune responses during the early phase of PRRSV infection could affect to the later immunological responses against the virus. Several studies suggested that the non-structural proteins (NSPs) and nucleocapsid protein (N-protein) of PRRSV could modulate Type I IFN production in an *in vitro* culture system (Lunney et al., 2016; Zhang et al., 2012). However, the immunosuppressive mechanism on the innate immune compartment of PRRSV during the early phase of infection remains incompletely understood.

This study aims to investigate the potential immunomodulatory mechanism related to suppression of the innate cytokines during the early phase of PRRSV infection. Interestingly, the preliminary microarray analysis demonstrated that PRRSV infected monocyte derived dendritic cell (MoDC) strongly upregulated interleukin-1 receptor antagonist (IL-1Ra) gene expression at 24 hours post-infection. IL-1Ra is known

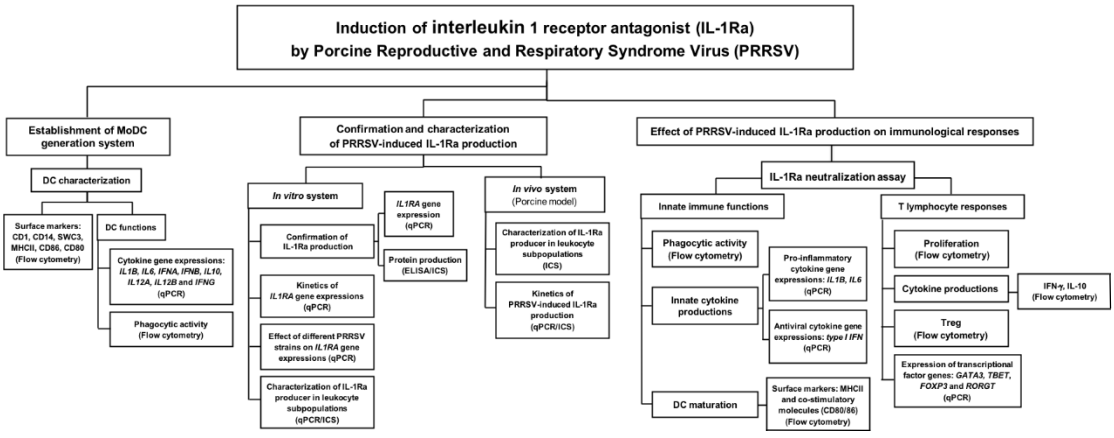
as an early anti-inflammatory cytokine, which controls inflammatory responses during the early immune activation cascade. IL-1Ra inhibits host immune responses by competitive binding to interleukin-1 receptor (IL-1R), which subsequently suppresses intracellular IL-1 signaling cascade (Arend, 2002; Arend et al., 1998). IL-1Ra is primarily produced from the cells of monocyte/macrophage lineage and DC, which are known as the major PRRSV target cells (Arend et al., 1998). In addition, it has been previously shown that IL-1Ra can suppress the production of IL-1 and TNF-**α** in macrophages (Marsh et al., 1994; Marsh and Wewers, 1994). The inhibition of IL-1 receptor signaling cascades is associated with regulation of type I IFN production (Mayer-Barber et al., 2014). It is possible that early IL-1Ra production could interfere induction of pro-inflammatory cytokines and type I IFNs during the early phase of PRRSV infection. We propose to investigate the effect of PRRSV on induction of IL-1Ra and to characterize the effects of PRRSV-induced IL-1Ra on the porcine immune functions.(Mayer-Barber et al., 2014) The obtained information will increase understanding in PRRSV immunopathogenesis and host-viral interactions.

1.2 Objectives

1. To modify previous conventional porcine monocyte-derived dendritic cell (MoDC) generation protocol
2. To confirm and characterize the induction of IL-1Ra production by PRRSV
3. To investigate the role of PRRSV induced IL-1Ra production on porcine innate immune functions
4. To investigate the role of PRRSV induced IL-1Ra production on porcine T cell responses

To fulfill these objectives, this dissertation present our findings into 5 separated chapters including chapter (1) Introduction and literature reviews, (2) Generation of potent porcine monocyte-derived dendritic cells (MoDC) by modified culture protocol, (3) Interleukin-1 receptor antagonist: an early immunomodulatory cytokine induced by porcine reproductive and respiratory syndrome virus, (4) Negative Immunomodulatory Effects of Porcine Reproductive and Respiratory Syndrome Virus-Induced Interleukin-1 Receptor Antagonist on Porcine Immune Responses and (5) General conclusion, discussion and future recommendations.

1.3 Experimental design



1.4 Literature review

1.4.1 Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) and its clinical outcomes

Porcine Reproductive and Respiratory Syndrome virus (PRRSV) is a major swine pathogen causing economic loss in the swine industry worldwide including Thailand. PRRSV is a member of the order *Nidovirales,* belongs to the family *Arteriviridae,* genus *Arterivirus*. PRRSV is an enveloped virus, 50-72 nm in diameter with an isometric core enclosing a linear positive-stranded RNA genome of approximately 15 kb (Rossow et al., 1995). The virus can be classified into two genotypes, type 1 (European; EU) and type 2 (North American; US) genotype, with considerable genetic variation within the genotypes (Brar et al., 2014; Wellenberg, 2006). The first PRRSV report in Thailand was in 1996, which was the US genotype. The clinical manifestations of PRRSV can be separated into two groups involving reproductive and respiratory systems resulting reproductive failure and respiratory disorders. PRRSV induces reproductive disorders in breeding herd, which is characterized by late-term abortion, mummification, increased stillborn, increased weak newborn piglets and affect the semen quality (Christopher-Hennings et al., 1998; Rossow, 1998). The virus also induces respiratory syndrome in nursery to finishing pigs, such as respiratory distress, viral pneumonia, and increased susceptibility to secondary infections associated with porcine respiratory disease complex (PRDC) (Beyer et al., 1998).

In 2006, the highly pathogenic PRRSV (HP-PRRSV) of atypical type II PRRSV, with deletion of 30 amino acids in the Non-structural protein 2 (Nsp2) gene, emerged in China (Tian et al., 2007). HP-PRRSV subsequently extended to Southeast Asia. In 2010, the first outbreak of HP-PRRSV in Thailand was reported (Nilubol et al., 2012). HP-PRRSV is characterized as a variant genotype of PRRSV, which shares genetically background with either type 1 or type 2 PRRSV (Zhou et al., 2011). The clinical manifestation of

HP-PRRSV is different from PRRSV, causes fatal diseases and associated with high morbidity and mortality rate in infected pig (Gao et al., 2015; Li et al., 2015). Recently, HP-PRRSV becomes an endemic strain in Thailand and other neighboring countries (Jantafong et al., 2015).

1.4.2 PRRSV cell tropism and generation of MoDC for studying of PRRSV immunobiology

Monocyte/macrophage or myeloid cell lineage mostly expresses heparan sulphate and sialoadhesin, which are critical for attachment of PRRSV on the target cells (Delputte et al., 2005). In addition, the scavenger receptor, CD163, abundantly expressed on myeloid cell lineage, is essential for viral genome release following viral attachment (Van Gorp et al., 2008). The finding suggested that PRRSV has very narrow cell tropism. The target cells of PRRSV are the cells of monocyte/macrophage lineage, dendritic cells (DC) in lung, lymphoid tissues and placenta (Welch and Calvert, 2010). Moreover, Macrophage precursor cells such as peripheral blood monocytes and bone marrow cells are also highly susceptible to PRRSV infection (Duan et al., 1997a, b; Teifke et al., 2001).

In the past, pulmonary alveolar macrophage (PAM), monocyte and DC were utilized for investigation of the roles of PRRSV on innate immune responses (Rodriguez-Gomez et al., 2013; Summerfield and McCullough, 2009). Unfortunately, there were limited by laborious isolation technique and amount of macrophage/DC population in blood circulation or tissues (Cohn and Delamarre, 2014; Summerfield et al., 2003). Therefore, *in vitro* DC generation was more practical and beneficial for studying of PRRSV-innate immunity interaction. At present, conventional porcine monocyte-derived DC (MoDC) culture protocol was preferentially used (Lecours et al., 2011; Wongyanin et al., 2012). However, the conventional MoDC generation technique requires high cell number and harvesting step prior to *in vitro* activation. During

harvesting step, pressure stimulation may accidentally affect to unwanted DC maturation that interferes result interpretation (Craig et al., 2009). In human and murine, conventional MoDC or BMDC was generated by different procedure, which has been validated and exhibited potent DC phenotypes (CD1, CD11C, MHCII and CD80/86) and theirs functions; phagocytosis and cytokine productions (Dewitte et al., 2014; Frindel et al., 1967; Qu et al., 2014). In this study, to overcome the problems, the *in vitro* culture procedure of conventional MoDC will be modified based on human MoDC and murine BMDC generation protocol.

1.4.3 The effect of PRRSV on immunocompetency of the infected host

During the early phase of PRRSV infection, high viral load can be detected in the circulation and lasts up to 1 month (Lopez and Osorio, 2004). Moreover, PRRSV can persist at a low level in lung tissues for 137-153 dpi (Chung et al., 1997). In addition, PRRSV can continuously replicate, at low level, in lymphoid organs for several months post infection (Beyer et al., 1998). PRRSV infected pigs, also predispose to secondary infections by other respiratory pathogens, known as porcine respiratory disease complex (PRDC), (Lunney et al., 2016). The interaction of PRRSV and bacteria in infected lung has been described that PRRSV could induce strong CD14 expression on monocyte/macrophage (Van Gucht et al., 2005). CD14 is known as a LPS binding receptor, which can induce the production of pro-inflammatory cytokines including IL-1β, IL-6, IL-8 and IFN-α. The finding indicated that PRRSV infection could enhance the severity of secondary bacterial infection due to overwhelmed of LPS stimulation in the infected lung (Van Gucht et al., 2005). In addition, PRRSV infection resulted in reduced classical swine fever virus (CSFV) vaccine efficacy (Suradhat et al., 2006), indicating that PRRSV infection could affect the host overall immunocompetency and caused vaccination failure.

1.4.4 Role of PRRSV on innate immunity

PRRSV primarily infects antigen presenting cells (APC) including macrophages and dendritic cells, which affect phagocytic ability and antigen processing in infected cells (Mateu and Diaz, 2008). PRRSV interferes antigen presentation by reduction of MHC I and MHC II expressions on macrophage and MoDC (Loving et al., 2007; Wang et al., 2007). Expressions of the co-stimulatory molecules; CD80 and CD86, are also compromised in PRRSV-infected MoDC (Rodriguez-Gomez et al., 2013). PD-L1 is known as an immunoregulatory ligand, which can induce apoptosis and T-cell anergy (Kuipers et al., 2006; Martin-Orozco et al., 2006). In earlier reports, PRSSV has an ability to enhance PD-L1 expression on dendritic cells, leading to the induction of Treg (Richmond et al., 2015). The findings suggested that PRRSV can modulate the phenotypes of APC as one of immune evasion strategies. Furthermore, the monocyte/macrophage and APC are responsible for production of several pro-inflammatory cytokines and anti-viral cytokines (Iwasaki and Pillai, 2014). Remarkably, the levels of innate cytokines produced following PRRSV infection, compared to other respiratory viruses, are usually slow and weak (Garcia-Nicolas et al., 2014; van Reeth and Nauwynck, 2000). During the early period of PRRSV infection, the virus can suppress production of type I IFNs, both IFN-α and IFN-β (Beura et al.; Kim et al.; Loving et al., 2007; Miller et al., 2004; Song et al.) and pro-inflammatory cytokines, including TNF-α, IL-1 and IL-6 (Garcia-Nicolas et al., 2014; van Reeth and Nauwynck, 2000). It has also been reported that PRRSV could suppress the production of IL-1β, IL-8 and IFN-α in serum during the first 14 dpi (Lunney et al., 2010). Consistent to the report in serum, reduction of IL-1, Il-6 and TNF-α productions were also observed in the infected lungs during the first 10 days of PRRSV infection (van Gucht et al., 2003).

In contrast to PRRSV, HP-PRRSV induces severe clinical manifestation and high mortality rate. Several studies suggested that the severity of disease in HP-PRRSV

infected pigs might be associated with the induction of strong pro-inflammatory responses. During the first week of infection, HP-PRRSV dramatically induced IL-1β, Il-6 and TNF-α production in serum (Liu et al., 2010). Moreover, the up-regulation of IL-1α production in lung tissue was associated to HP-PRRSV induced inflammatory lung lesions (Amarilla et al., 2015). The mechanisms of PRRSV induced different pro-inflammatory cytokine profiles remains unclear.

1.4.5 Role of PRRSV on adaptive immunity

Neutralizing antibodies block PRRSV infection by preventing the interaction of the virus with the receptor on monocyte/macrophage or APC (Lopez and Osorio, 2004). The studies on the humoral response against PRRSV indicated that viral-specific antibodies induced during the first 2 weeks following infection are non-neutralizing, while PRRSV-specific neutralizing antibodies appear quite late, up to 8 weeks post-infection (Nelson et al., 1994).

The role of cell-mediated immune (CMI) response is to protect intracellular pathogens and enhance phagocytic activity. The CMI is mediated by T lymphocytes, through helper T cells ($CD4^+$) and cytotoxic T cells ($CD8^+$) functions. The antigen-specific CMI response can be monitored by increased IFN-γ production against particular stimulus (Schoenborn and Wilson, 2007; Schroder et al., 2004). Interestingly, PRRSV-specific IFN-γ producing cells are usually not detected until 4 weeks post infection, which is very late, compared to other swine viruses (Lopez and Osorio, 2004; Olin et al., 2005). For example, classical swine fever vaccinated pigs developed viral-specific IFN-γ producing cells within 1 week post-vaccination (Suradhat et al., 2001). It has been reported that major PRSSV-specific IFN-γ producing cells are $CD4^+CD8^+$ cell, with a small proportion of $CD4^-$ $CD8^+$ cytotoxic T-cell (Diaz et al., 2005).

1.4.6 PRRSV immune evasion strategies

Generally, several viruses; such as swine influenza virus (SIV) and classical swine fever (CSFV) induce strong IL-1, IL-6, TNF-α and type I IFN production during 24 hours to 96 hours of infection (van Reeth and Nauwynck, 2000). Induction of innate cytokines is beneficial for early viral elimination and induction of pathogen-specific immune responses. In contrast to other swine viruses, PRRSV induced delayed and weak innate cytokines including TNF-α, IL-1, IL-6 and type I IFN during the first week of infection. Several studies indicated that PRRSV Nsp, including Nsp1 and Nsp2 and Nsp11, suppress Type I IFN production (Calzada-Nova et al., 2011; Charerntantanakul et al., 2006). Nsp1, Nsp2, and Nsp11 inhibit IRF3 signaling pathway in infected macrophage, leading to the inhibition of Type I IFN production (Kim et al.; Wang and Zhang, 2014). Moreover, Nsp2 also interferes induction of Type I IFN induced interferon-stimulated gene 15 (ISG15), which is known to inhibit PRRSV replication (Sun et al., 2012). Notably, the reduction of pro-inflammatory cytokines and Type I IFN in PRRSV infected pig is observed during 24 hours post infection. The immunomodulatory mechanisms of PRRSV during the early infection remained incompletely understood.

Several studies indicate that PRRSV can induce a potent a immunosuppressive cytokine, IL-10 (Suradhat and Thanawongnuwech, 2003; Suradhat et al., 2003) and viral specific Treg (Silva-Campa et al., 2009; Silva-Campa et al., 2012; Wongyanin et al., 2010). PRRSV induced IL-10 production in the infected lung from 3 dpi (Chung and Chae, 2003). In circulating leukocytes, upregulation of IL-10 gene in PRRSV infected pig was found from 5 dpi (Suradhat and Thanawongnuwech, 2003). The finding indicated that the induction of IL-10 and circulating Treg may relate to delayed adaptive immune responses in infected pig.

1.4.7 Function of innate cytokines on antiviral responses

Innate immunity is the first line defense mechanism, which responses immediately to invading microorganisms. Type I IFNs including IFN-α and IFN-β are produced from macrophages, plasmacytoid DC and infected tissues and can promote antiviral stage in infected cells. Pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α), produced by epithelium cells, phagocytic cells and APC, can promote cellular cross-talk, inflammation, leukocyte recruitment and shape the subsequent antigen-specific immune response (Iwasaki and Pillai, 2014).

1.4.7.1 Type I IFNs

The functions of type I IFNs include inhibition of viral replication, increase natural killer cell (NK cells) function, and expression of MHC Class I molecules on the virus-infected cells, and enhance Th1 cells development (Ivashkiv and Donlin, 2014). Type I IFNs receptor is widely expressed on several cell types (Theofilopoulos et al., 2005). After binding to its receptor, Type I IFNs induce intracellular transcriptional signals to promote interferon-stimulated gene expression (ISG), which is essential for induction of anti-viral stage and inhibition of viral replication in the targeted cells (Lauder et al., 2013). In addition, it has been reported that in influenza virus infection, type I IFNs are essential for induction of local B-cell responses and IgG productions. The failure of type I IFNs receptor stimulation affects elimination of influenza virus in the lungs (Coro et al., 2006).

1.4.7.2 Tumor necrosis factor-α (TNF-α)

Tumor necrosis factor-α (TNF-α), produced mainly by macrophages, stimulates the recruitment of neutrophils and monocytes to the site of infection and enhances expression of MHC Class I and Class II (Choi and Chae, 2002). TNF-α enhances expressions of the death receptors; Fas, TRAIL-R1 and TRAIL-R2 expression and promotes apoptotic cascade in virus-infected cells in cytomegalovirus (CMV), herpes

simplex virus (HSV) and adenovirus infection models (Benedict, 2003). In the influenza infection model, TNF-α induces strong type I IFNs production in lung epithelium cells, which is associated with early viral clearance (Seo and Webster, 2002). In hepatitis B virus infection model, TNF-α is required for induction of virus-specific cytotoxic T cells proliferation and is critical for reduction of hepatitis B antigen in infected livers (Kasahara et al., 2003).

1.4.7.3 Interleukin-6 (IL-6)

Interleukin-6 (IL-6), produced mainly by macrophages, mediates host inflammatory responses, recruitment of inflammatory cells to infective sites and stimulates the NK cells and T cell proliferation (Azuma et al., 2014; Oleksowicz and Dutcher, 1994). In influenza infection, IL-6 enhances neutrophil survival and limits the productions of inflammatory mediators (TNF-α, CCL2) in infected lungs, which are essential for viral clearance and protection of lethal lung pathology (Dienz et al., 2012; Lauder et al., 2013).

1.4.7.4 Interleukin-1 (IL-1)

Interleukin-1 (IL-1) is a potent pro-inflammatory cytokine, which has board properties in immunological responses. IL-1 has an ability to induce inflammatory mediators in affected tissue including cyclooxygenase type 2 (COX-2), type 2 phospholipase A, and inducible nitric oxide synthase (iNOS) accounts for prostaglandin-E2 (PGE2), platelet activating factor, and nitric oxide (NO) productions. IL-1 enhances adhesion molecules on targeted leukocytes, which recruits leukocytes from blood vessel into affected tissues. IL-1 can induce IL-6 production from monocytes/macrophages (Dinarello, 2009). In addition, combination of IL-1 and IL-18 enhances the expressions of CD40, OX40L (known as signalling lymphocytic activation molecules) and IL-12 production (Sims and Smith, 2010). These findings suggested that IL-1 promotes T cell stimulation. Notably, IL-1 is a co-stimulatory mediator for T helper

cell polarization and proliferation (Johnson et al., 2005). Moreover, IL-1 is also required for pathogen-specific B lymphocyte proliferation and T-cell-dependent antibody production (Nakae et al., 2001). The roles of IL-1 on viral elimination have been reported. For example, IL-1 inhibits hepatitis c virus (HCV) replication via induction of antiviral pathway; interferon-stimulated genes (ISGs), 1-8U in infected cells (Zhu and Liu, 2003). Furthermore, the combinations of IL-1 and complements (CR1 and CR2) induce local neutrophil activities to promote elimination of adenovirus-infected cell in liver (Di Paolo et al., 2014).

Together, the information indicates that the poor innate immune responses may affect to adaptive immune functions, leading to failure to pathogen elimination (Iwasaki and Pillai, 2014).

1.4.8 IL-1Ra: potential immunomodulatory factor during the early phase of PRRSV infection

Interleukin-1 receptor antagonist (IL-1Ra) is known as an inhibitory cytokine, which plays a role during the early phase of immune responses. IL-1Ra belongs to the interleukin-1 (IL-1) family, and has different conformation structure, compared to the IL-1α or IL-1β (Arend et al., 1998). The interaction of IL-1 and IL-1 receptor (IL-1R) enhances inflammatory responses via induction of downstream IL-1R signaling cascades. IL-1Ra can competitively bind to IL-1R with high avidity, resulting in inhibition of IL-1R signaling pathways (Arend, 2002). Monocyte, macrophage, DC and neutrophil subpopulations are responsible for IL-1Ra production (Arend et al., 1998).

1.4.8.1 Role of IL-1Ra on innate immunity

IL-1Ra had been reported to suppress productions of pro-inflammatory cytokines including IL-1 and TNF-α in monocytes/macrophages (Granowitz et al., 1992; Marsh et al., 1994). In keratinocytes, IL-1Ra inhibits COP9 signalosome signaling pathway, leading to suppression of IL-1α-induced IL-6 and IL-8 productions (Banda et al., 2005). In a

mouse model, IL-1Ra can modulate type I IFN production in lungs (Mayer-Barber et al., 2014). Furthermore, lack of IL-1 receptor signaling pathway exhibits the subversion of bacterial-induced type I IFN production (Ludigs et al., 2012; Mayer-Barber et al., 2014). In case of pneumonic plague (*Yersina pestis* infection), IL-1Ra can suppress IL-1/IL-8-induced hyperinflammation in early phase of infection (Sivaraman et al., 2015). Previously, synthetic IL-1Ra peptide has been used for treatment of rheumatiod arthritis (RA) and inhibits IL-1, IL-6 and TNF-α productions in RA patients (Schiff, 2000).

1.4.8.2 Role of IL-1Ra on adaptive immunity

In a mouse infectious model, synthetic IL-1Ra could inhibit T-helper cell polarization, in particular T-helper 2 and T-helper 17 (Nakae et al., 2003; Wang et al., 2006). In a mouse autoimmune model, the imbalance of IL-1/IL-1Ra could affect T-lymphocyte activation and proliferation (Matsuki et al., 2006). In addition, lack of IL-1 receptor signaling cascade could reduce the production of antibodies against the specific antigen (Nakae et al., 2001). In herpes virus infection, overexpression of IL-1Ra production reduces the resident $CD4^+$ cells and suppresses IL-6 and macrophage-inflammatory protein-2 (MIP-2) productions in infected tissues (Biswas et al., 2004). The finding suggests that IL-1Ra production also affects to the induction of adaptive immune responses.

Interestingly, our preliminary A microarray analysis demonstrated that PRRSV infected monocyte-derived dendritic cells (MoDC) exhibited strong upregulation of IL-1Ra gene expression at 24 h post-infection. The information suggests that IL-1Ra may play a role in innate cytokines suppression in the early phase of PRRSV infection and explain the difference of pro-inflammatory cytokines production following PRRSV and HP-PRRSV infection.

CHAPTER II

Generation of potent porcine monocyte-derived dendritic cells (MoDC) by modified culture protocol

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Generation of potent porcine monocyte-derived dendritic cells (MoDC) by modified culture protocol

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(Appendix A)

Teerawut Nedumpun, Patcharee Ritprajak and Sanipa Suradhat

Generation of monocytes-derived dendritic cells (MoDC) are utilized as an *in vitro* assay for studying immunopathogenesis of porcine viruses. In this chapter, we modified the previous conventional porcine MoDC generation protocol to improve the quality of the MoDC. Our finding provided a reliable constant porcine MoDC generation protocol for the future *in vitro* investigation.

2.1 Abstract

In vitro derivation of dendritic cells (DC) is an alternative approach to overcome the low frequency of primary DC and the difficulty of isolation techniques for studying DC immunobiology. To date, the conventional culture protocol of porcine monocyte-derived DC (MoDC) has been widely used. However, this protocol is not practical due to the requirement of a substantial number of blood monocytes, and the process often interferes with DC maturation. To improve *in vitro* porcine MoDC generation, we modified the previous conventional DC generation protocol, based on the human and mouse primary DC culture system, and compared phenotypic and functional features of MoDC derived from the modified protocol to the conventional protocol. The modified protocol consumed fewer monocytes but generated higher $CD1^+$ cells with DC-like morphology and the ability of maturation. In addition, MoDC from the modified protocol exhibited increased antigen uptake and IFN-γ production in response to LPS stimulation. Our findings indicate that the modified protocol is expedient and reliable for generating potent MoDC that substitute for primary DC. This will be a valuable platform for future research in antigen delivery, vaccines and immunotherapy in pigs, as well as relevant veterinary species.

Keywords: Dendritic cell, Porcine monocyte derived-dendritic dendritic cells, Conventional protocol, Modified protocol, *In vitro* culture

2.2 Introduction

Dendritic cells (DC) are a unique innate immune cell capable of antigen processing and presentation, and they also provide the signal for T lymphocyte activation and differentiation (Merad et al., 2013). Knowledge of the immunomodulatory properties of DC could lead to improvements in the field of swine vaccinology and immunotherapy. Porcine DC have been characterized and typified by the expression of the surface molecules, CD1, CD14, CD16, CD80/86, CD172a and MHC class II (Summerfield and McCullough, 2009). However, study of porcine DC biology is limited due to the low frequencies of DC populations in blood circulation and tissues, and laborious DC isolation techniques (Summerfield and McCullough, 2009; Zhang et al., 2009). Therefore, *in vitro* monocyte-derived DC (MoDC) is an alternative tool to examine porcine DC biological properties and functions.

Two different *in vitro* culture procedures for porcine DC generation from peripheral blood monocytes have been established. The fast protocol can generate porcine MoDC, so-called fast MoDC, within 48 h, but the phenotype and function of these DC are only partially developed (Wongyanin et al., 2010). The conventional protocol requires 7–9 days to induce MoDC, but these DC are more potent and favorable for investigating the host- pathogen interaction (Lecours et al., 2011; Summerfield and McCullough, 2009). However, there is a limitation of the conventional protocol, as it requires a large amount of peripheral blood mononuclear cells (PBMC). In addition, the protocol includes cell harvesting and re-plating steps prior to *in vitro* stimulation which may possibly induce undesirable DC maturation by physical and pressure stimulation (Banchereau et al., 2000). The biological and functional properties of immature and mature DC are different. Immature DC are capable of high antigen uptake but low T lymphocyte activation properties, while mature DC lose their phagocytic ability but can mediate T lymphocyte activation and differentiation (Schnurr

et al., 2000). Therefore, the stage of DC maturation is crucial for the downstream experimental interpretation.

The standard protocol for generating *in vitro* DC in humans and mice are quite similar and have been well validated (Dewitte et al., 2014; Frindel et al., 1967; Inaba et al., 2009; Qu et al., 2014). Generations of human MoDC and murine bone marrow-derived DC (BMDC) requires fewer DC precursors than the porcine MoDC protocol. Furthermore, the harvesting and re-plating steps used in the porcine system are generally excluded from the human and mouse systems. Due to the aforementioned disadvantages of the current porcine MoDC generation protocol, our study aimed to improve a porcine MoDC generation protocol by modifying the current conventional protocol, referred to as the modified protocol, based on human MoDC and murine BMDC culture procedures. This work will be beneficial for the study of immunobiology of porcine DC and mechanisms of host-pathogen interactions. The validated MoDC will also be expedient for future research works in antigen delivery, vaccines and immunotherapy in pigs, as well as relevant species.

2.3 Materials and Methods

2.3.1 Animals and blood collection

Eight-week-old, crossbred pigs were subjected to whole blood collection. The heparinized blood samples were kept in at 4°C during transportation and processed within 4 h following blood collection. All animal procedures were reviewed and approved by Chulalongkorn University Animal Care and Use Committee (protocol number 1631029).

2.3.2 *In vitro* generation and stimulation of porcine MoDC

Porcine PBMC were isolated from heparinized whole blood by density gradient centrifugation using Lymphosep® separation medium (Biowest, Kansas, Mo, USA) according to previously described protocol (Suradhat and Thanawongnuwech, 2003).

For the conventional protocol, 25×10^6 porcine PBMC were plated in 1ml of IMDM containing 5% fetal bovine serum (FBS) in a well of 6-well-plate for 2 h, followed by removal of the non-adherent cells. The adhered cells, referred as monocytes, were subsequently cultured with 1 ml of RPMI containing 10% FBS, 2 mM L-glutamine, antibiotic/antimycotic solution, 25 mM HEPES (all above reagents were obtained from GIBCO, Carlsbad, CA, USA) and 50 µM β-mercaptoethanol (Sigma Chemical Co., St. Louis, MO, USA), and supplemented with 10 ng/ml and 25 ng/ml recombinant porcine IL-4 and GM-CSF (R&D system, Minneapolis, MN, USA) respectively, referred as DC medium, for 7 days. Every 2 days, the culture medium was 50% replaced with fresh DC medium. At day 7, MoDC were harvested and re-plated for 1×10^6 per ml of DC medium in a well of 24-well-plate prior to DC activation experiment.

For the modified protocol, 5×10^6 PBMC in 1 ml of IMDM containing 5% FBS were plated in a well of 24-well-plate. Non-adherent cells were removed, and the adhered cells were further cultured with DC medium for 7 days. Every 2 days, the

culture medium was 50% replaced with fresh DC medium. For downstream *in vitro* studies, the MoDC were incubated with stimulus without harvesting and re-plating step. The cells from both protocols were cultured at 37°C in a 5% CO_2 incubator. For LPS stimulation, MoDC were stimulated with 0.1 mg/ml LPS (Sigma aldrich) for 24 h. Un-stimulated MoDC were used as the negative control.

Table 2.1 comparison of conventional and modified protocol

Culture protocol	**Conventional**	**Modified**
Number of initial PBMC per well	25×10^6 cells	5×10^6 cells
Culture ware	6-well plate	24-well plate
Volume of medium	1ml	1ml
Cytokines	25 ng/ml GM-CSF 10 ng/ml IL-4	25 ng/ml GM-CSF 10 ng/ml IL-4
Incubation time	7 days	7 days
Harvesting step	Yes	No
Re-plating step	Yes	No

2.3.3. Percentage of adhered monocyte and number of adhered MoDC

Following PBMC plating and removal of non-adherent cells, the remaining adhered cells were harvested and enumerated using Z2 Coulter counter (Beckman Coulter, CA, USA). Percentage of the cells was calculated by (number of the adhered cells/total number of PBMC) × 100. In addition, the 7-day cultured MoDC were harvested and examined the cell number using the same protocol.

2.3.4 MoDC morphology

The morphology of cultured monocytes and MoDC at day 7 were observed under the inverted bright field microscopy (Olympus), at 40× stage objectives.

2.3.5 Phagocytosis assay

The phagocytosis assay was performed as previously described (Dewitte et al., 2014). MoDC (5×10^5 cells/well) were incubated with FITC-conjugated Escherichia coli (Molecular Probes, Invitrogen, USA) at 1:50 ratio of MoDC : *E. coli*, at 37°C for 10 min with shaking. Then, cold PBS was added to stop phagocytic activity, and the cells were washed 4 times. The MoDC were then resuspended in FACS buffer for flow cytometric analysis.

2.3.6 Flow cytometric analyses

For surface markers staining, Primary antibodies included anti-porcine CD1-PE (76-7-4), anti-porcine SLA-DR (1053H2-18), biotinylated-anti-porcine CD172a (74-22-15) were obtained from SouthernBiotech (Birmingham, AL, USA), and anti-human CD86-PEcy7 (IT2.2) and the isotype control antibody (MPC-11) were obtained from BioLegend (San Diego, CA, USA). Secondary antibodies including goat anti-mouse-FITC, IgG2a and streptavidin-APC were obtained from Invitrogen (Carlsbad, CA, USA). For intracellular staining, MoDC were stained with biotinylated-anti-porcine IFN-γ (P2C11, IgG2a) (BD Biosciences, San Diego, CA, USA) and streptavidin-PE/cy7, or IgG2a and isotype control (BioLegend). Subsequently, the stained cells were analysed by Beckman FC550 (Beckman Coulter), and FlowJo software.

2.3.7 Quantitative real-time polymerase chained reaction (qRT-PCR)

Following LPS stimulation, total mRNA was extracted from the MoDC using UPzol™ (Biotechrabbit, Germany), and converted to cDNA by Supercript III First-Strand cDNA synthesis kit (Invitrogen). The qRT-PCR reaction was performed as previous described (Wongyanin et al., 2012). The specific primers used were *IL1B* (IL-1β), F 5′ AAC-GTG-CAA-TGA-TGA-CTT-TG 3′ and R 5′ CAC-TTC-TCT-CTT-CAA-GTC-CC 3′; *IL6* (IL-6), F 5′AGA-ACT-CAT- TAA-GTA-CAT-CCT-CG 3′ and R 5′ AGA-TTG-GAA-GCA-TCC-GTC 3′; *IL12A* (IL-12p35), F 5′ ATG-CCT-CAA-CCA-CTC-CCA-AA 3′ and R 5′ GGC-AAC-TCT-CAT-TCG-TGG-CT 3′; *IL12B* (IL-12p40), F 5′ CCC-TGG-AGA-AAT-GGT-GGT-CC 3′ and R 5′ GGC-CAG-CAT-CTC-CAA-ACT-CT 3′; *IFNA* (IFN-α), F 5′ CAC-CAC-AGC-TCT-TTC-CAT 3′ and R 5′ CAC-CAC-AGC-TCT-TTC-CAT 3′; *IFNB* (IFN-β), F 5′ CTG-GAG-GAG-GAC-TCC-AT 3′ and R 5′GAG-TCT-GTC-TTG-CAG-GTT 3′; *IFNG* (IFN-γ), F 5′ GAG-GTT-CCT-AAA-TGG-TAG-CTC 3′ and R 5′ GTC-TGA-CTT-CTC-TTC-CGC 3′; *GAPDH* (GAPDH), F5′ AAG-TGG-ACA-TTG-TCG-CCA-TC 3′ and R 5′ TCA-CAA-ACA-TGG- GGG-CAT-C 3′. The Ct values of the target gene were normalized against those of the housekeeping gene; *GAPDH*. Differences in Ct values between the treatments were analysed by the formula $2^{-(\Delta Ct^{\text{target gene}} - \Delta Ct^{\text{GAPDH}})}$ using a Rotor-Gene Real-Time Analysis Software 6.0 (Corbett Research).

2.4 Results

2.4.1 The modified MoDC generation protocol increased the number of adherent monocytes, but still maintained dendritic cell-like morphology

The generation of MoDC by the conventional and modified culture procedures differed in two steps: the initial plating and re-plating (Table 2.1). At the initial step, the PBMC number in the modified protocol was 5-fold less than the conventional protocol; however, the percentage of adherent monocytes in the modified protocol was obviously greater than those in the conventional protocol (Fig. 2.1A). During the culture period, the cells from both procedures retained approximately 70–80% attachment (Fig. 1B). Compared with the original protocol, the modified protocol required fewer PBMC to generate the same amount of MoDC (Fig. 2.1A–B). After the 7-day-culture, the number of MoDC generated from the modified protocol was consistent among the individual wells (Fig. 2.1C), indicating that the harvesting and re-plating steps are dispensable for the MoDC culture.

The morphology of porcine MoDC has been well characterized by cytoplasmic projections around the cell surface (Lecours et al., 2011; Lin et al., 2012). We therefore observed the change in cell morphology at the end of the culture period. All MoDC elongated and transformed to DC-like morphology (Fig. 2.1Db–c and De–f), which was absent in the monocytes (Fig. 2.1Da, d). In addition, MoDC from the modified protocol (Fig. 2.1Dc, f) were phenotypically similar to those from the conventional protocol (Fig. 2.1Db, e), both in size and morphology.

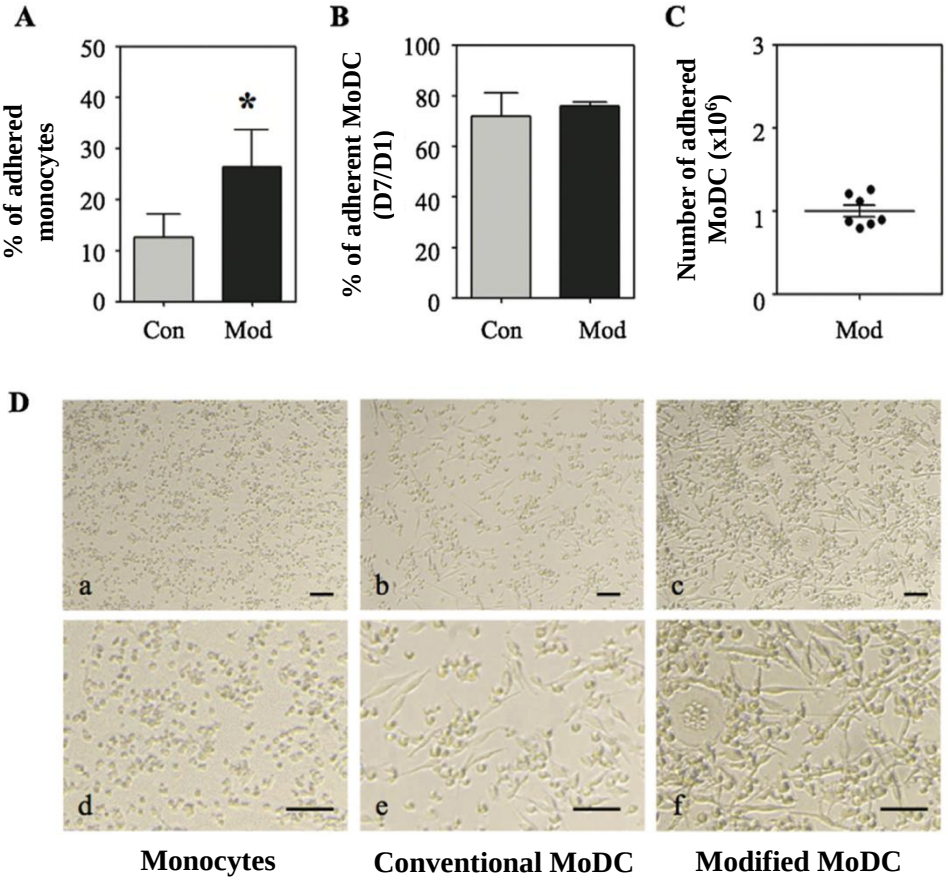

Figure 2.1 Percentage of adhered monocytes and morphology of monocytes and MoDC. (A) The percentage of adhered monocytes prior to MoDC induction in the conventional and modified protocol was determined by calculating the ratio of adhered monocytes per total PBMC. (B) MoDC were generated from two protocol and the proportion of adherent MoDC on day 7 over day 1 was calculate as the percentage. (C) Total number of 7-day cultured MoDC generated by the modified protocol from seven individual samples. * $p<0.05$ comparing between MoDC from both protocols. Con, conventional protocol; Mod, modified protocol. (D-a, d) Porcine peripheral blood monocytes were cultured in non-DC medium and MoDC generated from the (D-b, e) conventional and (D-c, f) modified protocols were cultured in DC medium. At day 7, cell morphology was observed under bright field microscopy (40x). Scale bars, 50 μm.

2.4.2 MoDC generated from the modified protocol highly expressed the dendritic cell marker, CD1 molecule, and had increased phagocytic activity

To characterize phenotypes of the obtained MoDC, surface expressions of CD1, SLA-DR and CD172a markers were determined (Fig. 2.2A–B). The CD1 molecule has been recognized as a lipid presentation molecule solely expressed in the porcine DC population (Summerfield and McCullough, 2009). Notably, MoDC from the modified protocol highly expressed CD1 molecules, while the MoDC from the conventional protocol expressed CD1 at a comparable level to the monocytes (Fig. 2.2A–C, left panel). Furthermore, the $CD1^+$ population was markedly presented in MoDC from the modified protocol but was only moderately present in MoDC obtained from the conventional protocol (Fig. 2.2A–C, left panel). The obtained MoDC from both protocols showed upregulated expression of SLA-DR and CD172a and an elevated percentage of SLA-DR^+ and $CD172a^+$ populations when compared to the monocytes (Fig. 2.2B–C, middle and right panels). However, there was no statistical difference in the SLA-DR and CD172a expression level and SLA-DR^+ and $CD172a^+$ cells between the two protocols (Fig. 2.2B–C, middle and right panel).

To confirm whether these DC were able to function, we assessed phagocytosis activity by using FITC-labeled *Escherichia coli* as an antigen. Comparing between the two culture protocols, MoDC derived from the modified protocol displayed a higher efficacy of *E. coli* engulfment (Fig. 2.2D).

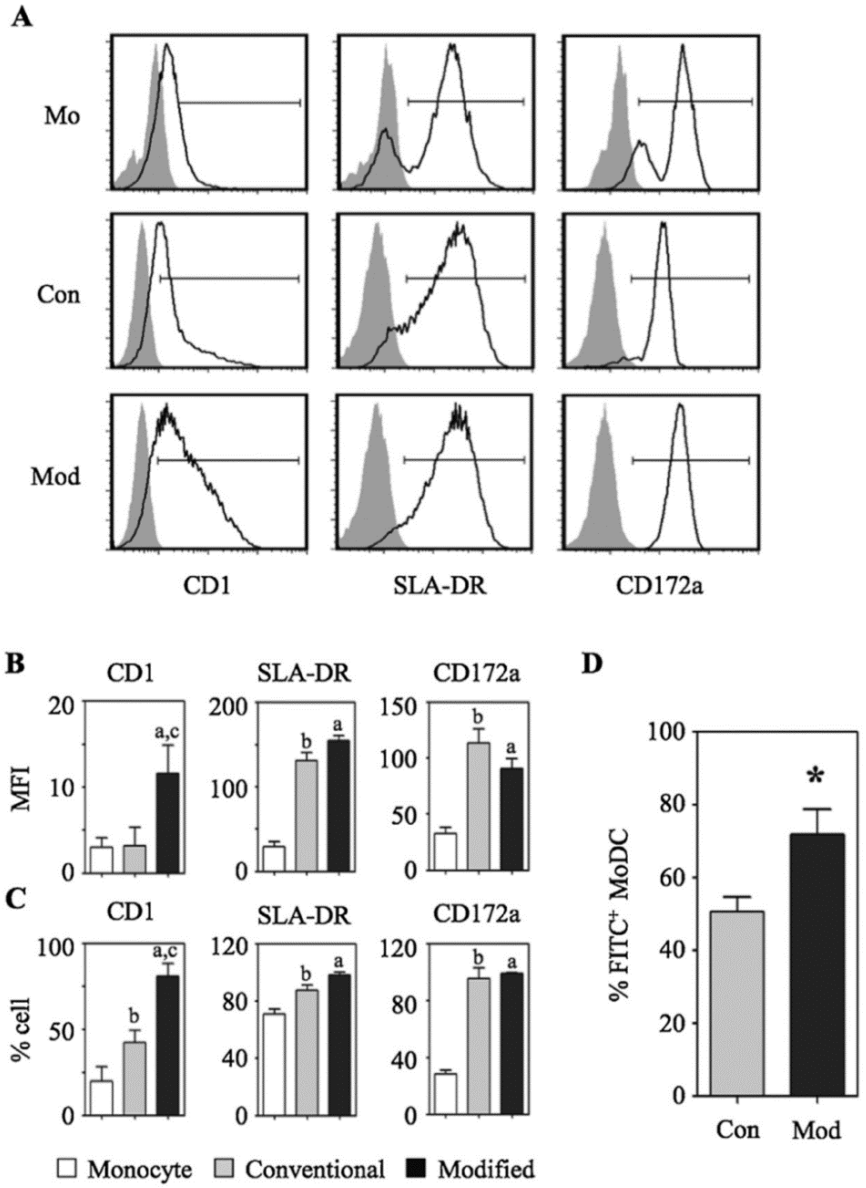

Figure 2.2 Comparative analysis of MoDC phenotype and phagocytic activity. Monocytes and immature MoDC generated from the modified and conventional protocols were harvested at day 7. (A–C) The cells were stained against CD1, CD172a and SLA-DR and analysed by flow cytometry. (A) Histogram demonstrating the expression level of DC marker (thick line) and isotype control staining (gray shade). Data are representative of three independent experiments. (B) Mean fluorescent intensity (MFI) and (C) the percentage of positive cells of CD1, CD172a and SLA-DR in monocytes and MoDC from the conventional and modified protocols were determined. (D) MoDC

obtained from each protocol were cultured with FITC-labeled *E. coli*. The percentage of FITC positive cells, representing MoDC-engulfing *E. coli*, were analysed by flow cytometry. [a] $p<0.05$ when comparing MoDC from the modified protocol to monocytes; [b] $p<0.05$ when comparing MoDC from the conventional protocol to monocytes; [c] $p<0.05$ when comparing MoDC from the modified protocol to those of the conventional protocol. * $p<0.05$ when comparing MoDC from the modified protocol to those of the conventional protocol; n = 5 pigs. Mo, monocytes; Con, conventional protocol; Mod, modified protocol. Two different MoDC generation protocols were performed concurrently using PBMC from the same animals. Data are representative of two independent experiments.

2.4.3 MoDC generated from modified protocol were capable of undergoing maturation

According to the efficient DC differentiation obtained from the modified protocol, we next questioned whether these MoDC were able to undergo maturation. To evaluate the DC maturation stage, the cultured MoDC were stimulated with LPS, and the expression of maturation markers SLA-DR, CD80 and CD86 were determined (Fig. 2.3). From flow cytometric analyses, all MoDC altered their phenotype upon LPS stimulation by upregulating the expression of SLA-DR and CD86, but not CD80, molecules (Fig. 2.3A–C). The frequency and level of maturation markers in MoDC from the modified protocol were similar to those from the conventional protocol (Fig. 2.3B and C). The data suggests that our modified culture procedure was capable of generating potent MoDC *in vitro*.

2.4.4 The modified protocol improved effector properties of mature MoDC

Generation of porcine MoDC is not only beneficial for the study of porcine DC biology but is also essential for *in vitro* tests in clinical application. *In vitro* generated DC, therefore, must represent *in vivo* DC in both cellular phenotype and effector properties. To investigate the effector properties of mature MoDC, the MoDC were stimulated with LPS and the expression of pro-inflammatory cytokine genes (Fig. 2.3D) was determined. Upon LPS stimulation, *IL1B*, *IL6* and *IFNB* mRNA, encoding IL-1β, IL-6 and IFN-α, respectively, were induced in MoDC generated from the modified protocol at levels comparable to those from the conventional protocol. Interestingly, the expression level of the *IFNG* gene, encoding IFN-γ, was significantly upregulated in MoDC obtained from the modified protocol (Fig. 2.3D). To confirm the production of IFN-γ protein levels, LPS-stimulated MoDC were subjected to flow cytometric analyses. Consistent with results from the gene expression study, upon LPS stimulation, the level of IFN-γ production was greatly increased in both bulk and $CD1^+$ in MoDC obtained from the modified protocol, while IFN-γ production in the MoDC obtained from the conventional protocol was only slightly increased (Fig. 2.3E).

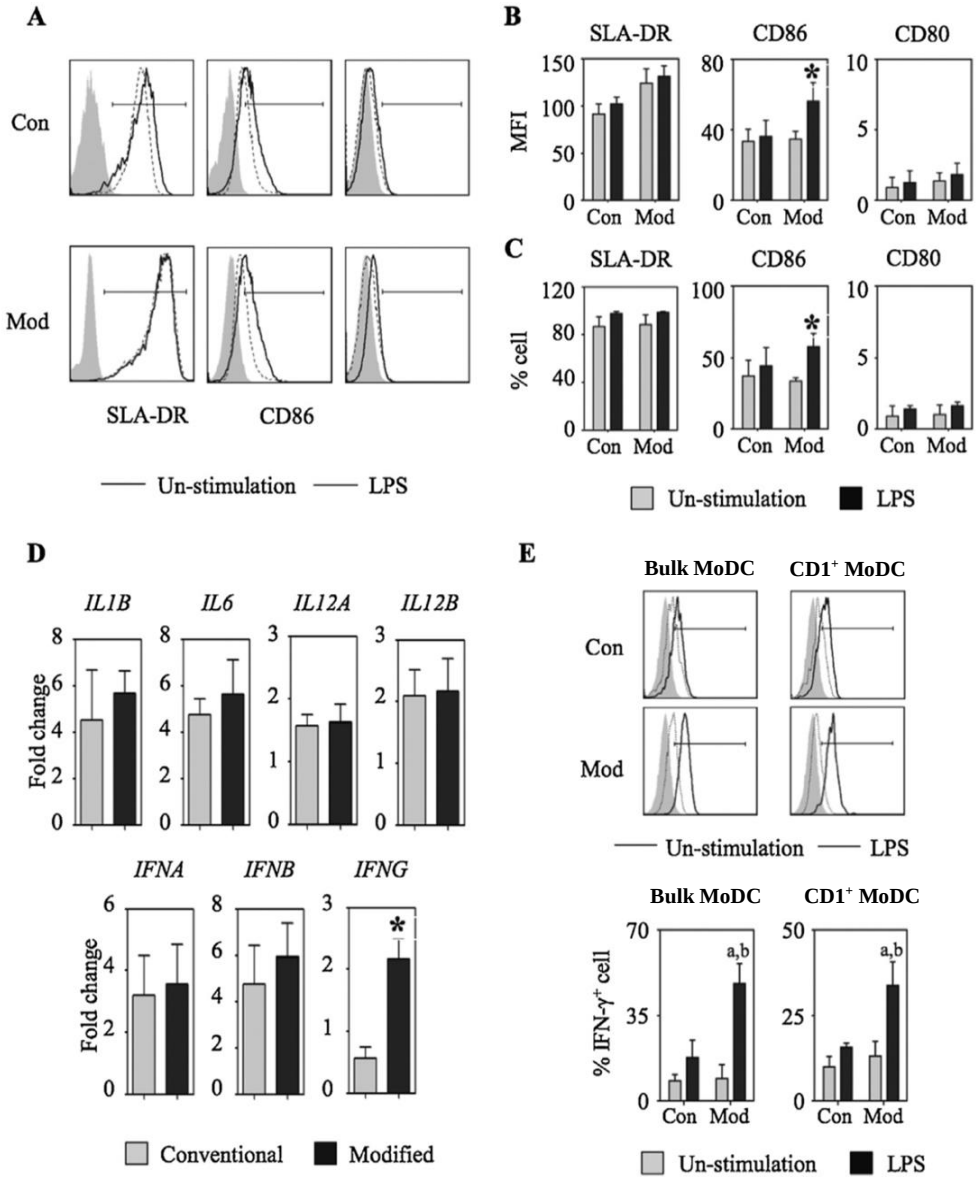

Figure 2.3 The expressions of DC maturation markers and pro-inflammatory cytokines. MoDC generated from the conventional and modified protocols were stimulated with 1 μg/ml LPS for 24 h. (A–C) The cells were stained for SLA-DR, CD80 and CD86 and subjected to flow cytometric analyses. (A) Histograms demonstrating the expression level of the activation marker from LPS stimulation (thick line) and non-stimulated (dashed line) MoDC. Isotype control was used for the negative staining (grey shade). Data are representative of three independent experiments. The (B) mean fluorescent intensity (MFI) and (C) percentage of positive cells of SLA-DR, CD80 and

CD86 in monocytes and MoDC from the conventional and modified protocols were determined. (D) The expressions of the *IL1B*, *IL6*, *IL12A*, *IL-12B*, *IFNA*, *IFNB* and *IFNG* genes were evaluated by qRT-PCR. The relative gene expression was shown as the fold induction over the non-stimulated MoDC. * $p<0.05$ when comparing LPS stimulated MoDC from the modified protocol to those of the conventional protocol. (E) Histogram demonstrating the level of IFN-γ expression and isotype control staining (gray shade). The percentage of IFN-γ producing cells was determined by flow cytometric analysis from bulk MoDC and $CD1^+$ MoDC populations. a $p<0.05$ when comparing LPS stimulated MoDC to non-stimulated MoDC from the conventional protocol and b $p<0.05$ when comparing LPS stimulated MoDC from the modified protocol to those of the conventional protocol; n = 3 pigs. Con, conventional protocol; Mod, modified protocol. Two different MoDC generation protocols were performed concurrently using PBMC from the same animals. Data are representative of two independent experiments.

2.5 Discussion

The development of MoDC in the culture system provides great advantages to studies involving DC immunobiology, especially regarding the host-pathogen interaction. Here, we proposed an *in vitro* modified MoDC generation protocol that required fewer porcine PBMC and yielded a good quantity of porcine MoDC. The lower number of PBMC in the cultured system might improve the ratio of cells to contact surface area on the culture plate leading to enhanced monocyte adherence. As the downstream signals and the optimal doses of GM-CSF and IL-4 are mandatory for MoDC differentiation *in vitro* (Heystek et al., 2000; Menges et al., 2005), it is possible that the lower number of cultured monocytes per well in the modified protocol allowed better utilization of the supplements, leading to the higher quantity and quality of the generated porcine MoDC.

In addition, the re-plating step was excluded from our modified protocol. The purpose of re-plating MoDC in the conventional protocol was to control the accuracy of cell numbers, however, these steps may induce premature DC differentiation as a consequence of mechanical stimulation (Craig et al., 2009) leading to reduced phagocytosis ability and cytokine production in response to stimuli (Mellman and Steinman, 2001), which could affect the accuracy and reliability of the downstream experiment. The omitted mechanical stimulation in the modified protocol could have contributed to the more efficient DC generation.

The MoDC obtained from the modified protocol expressed the DC markers, CD1, CD172a and SLA-DR, similar to those generated by the conventional protocol, concordant with previous reports (Chamorro et al., 2005; McCullough et al., 1997; Summerfield and McCullough, 2009). CD1 is not constitutively expressed by monocytes but is upregulated during monocyte differentiation into DC (Summerfield and McCullough, 2009). Phagocytosis is a hallmark of DC functions that initiates antigen

processing and peptide presentation (Savina and Amigorena, 2007). The modified protocol increased number of $CD1^+$ cells and enhanced phagocytic activities indicating a more efficient generation of porcine MoDC by the modified protocol.

Of interest, MoDC generated by the modified protocol displayed high levels of *IFNG* gene expression and IFN-γ production upon maturation. IFN-γ is not only important for Th1 polarization but is also essential for driving DC maturation and endowing the effector properties of DC (Frasca et al., 2008; Han et al., 2009; He et al., 2007; Pan et al., 2004). Dendritic cells produce high levels of IFN-γ in response to stimuli, especially LPS (Fedele et al., 2008; Pan et al., 2004). Interferon- gamma receptor deficient BMDC exhibited decreased expression of the maturation marker, CD86, reduced production of the pro-inflammatory cytokines, IL-1β and IL-12, and impaired function to activate alloreactive T cells (Pan et al., 2004). In addition, exogenous IFN-γ enhanced DC maturation and function (Frasca et al., 2008; Han et al., 2009; Moretto et al., 2007). Interferon-gamma producing DC potentially induced the antigen-specific cytotoxic T cell responses both in vitro and in vivo (He et al., 2007; Lemoine et al., 2010; Moretto et al., 2007). Interferon-gamma thus acts as an autocrine mediator for DC maturation and exerts T cell effector functions.

In conclusion, our findings suggest that the modified protocol improved MoDC maturation and effector properties. The modified protocol has potential for numerous future applications and will be a valuable platform for future research in antigen delivery, vaccines and immunotherapy in pigs, as well as relevant veterinary species.

CHAPTER III

Interleukin-1 receptor antagonist: an early immunomodulatory cytokine induced by porcine reproductive and respiratory syndrome virus

This work has been published in the topic of

Interleukin-1 receptor antagonist: an early immunomodulatory cytokine induced by porcine reproductive and respiratory syndrome virus

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(Appendix B)

Teerawut Nedumpun, Piya Wongyanin, Chaitawat Sirisereewan, Patcharee Ritprajak, Tanapat Palaga, Roongroje Thanawongnuwech and Sanipa Suradhat

Porcine reproductive and respiratory syndrome virus (PRRSV) weakens innate immune responses in an early period of infection. Our microarray analysis demonstrated strong upregulation of the *IL1RA* gene in type 2 PRRSV-infected monocyte-derived dendritic cells (MoDC). In this chapter, we confirmed PRRSV-induced IL-1Ra production in both *in vitro* and *in vivo*. Interestingly, type 2 PRRSV and HP- PRRSV exhibited variation in their capability in regard to induction of IL-1Ra. Our findings could partly explain the differences in immunopathogenesis and clinical outcomes following PRRSV infections.

3.1 Abstract

Porcine reproductive and respiratory syndrome virus (PRRSV) infection poorly induces pro-inflammatory cytokines (IL-1, IL-6 and TNF-α) and type I IFN production during the early phase of infection. Our microarray analysis indicated strong upregulation of the *IL1RA* gene in type 2 PRRSV-infected monocyte-derived dendritic cells. Interleukin-1 receptor antagonist (IL-1Ra) is an early inhibitory cytokine that suppresses pro-inflammatory cytokines and T lymphocyte responses. To investigate the induction of IL-1Ra by PRRSV, monocyte-derived dendritic cells were cultured with type 2 PRRSV or other swine viruses. PRRSV increased both *IL1RA* gene expression and IL-1Ra protein production in the culture. The enhanced production of IL-1Ra was further confirmed in PRRSV-cultured PBMC and PRRSV-exposed pigs by flow cytometry. Myeloid cell population appeared to be the major IL-1Ra producer both *in vitro* and *in vivo*. In contrast to the type 2 PRRSV, the highly pathogenic (HP)-PRRSV did not upregulate *IL1RA* gene expression *in vitro*. To determine the kinetics of PRRSV-induced *IL1RA* gene expression in relation to other pro-inflammatory cytokine genes, PRRSV-negative pigs were vaccinated with a commercially available type 2 modified-live PRRS vaccine or intranasally inoculated with HP-PRRSV. In modified-live PRRS-vaccinated pigs, upregulation of *IL1RA*, but not *IL1B* and *IFNA*, gene expression was observed from 2 days post-vaccination. Consistent with the *in vitro* findings, upregulation of *IL1RA* gene expression was not observed in the HP-PRRSV-infected pigs throughout the experiment. This study identified IL-1Ra as an early immunomodulatory mediator that could be involved in the immunopathogenesis of PRRSV infections.

Keywords: PRRSV, HP-PRRSV, microarray, IL-1Ra, Myeloid cells, infected pigs

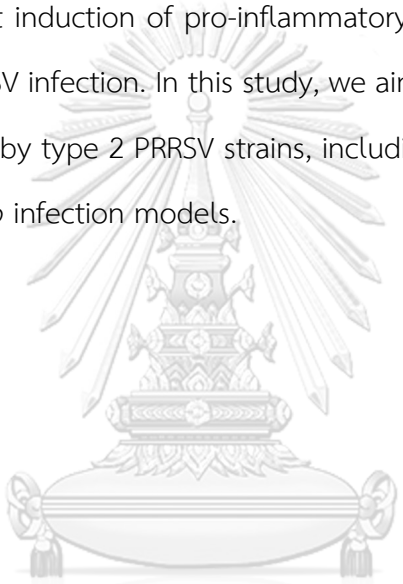
3.2 Introduction

Innate immunity is critical for induction of virus-specific immune responses (Iwasaki and Pillai, 2014). Pro-inflammatory cytokines (IL-1, IL-6 and TNF-α) and antiviral cytokines (type I interferon) are innate cytokines, which help promote leukocyte recruitment and inhibition of viral replication (Benedict, 2003; Dienz et al., 2012; Oleksowicz and Dutcher, 1994; Sims and Smith, 2010; Theofilopoulos et al., 2005). Porcine reproductive and respiratory syndrome virus (PRRSV) is one of the major swine pathogens that causes serious economic losses in the swine production industry worldwide (Rossow, 1998). PRRSV can be classified into two genotypes: type 1 (EU) and type 2 (US) strains (Meng et al., 1995). PRRSV infection usually induces poor innate and adaptive immune responses (Butler et al., 2014; Lunney et al., 2016). In addition, PRRSV infection usually enhances secondary infections in the lungs, leading to porcine reproductive and respiratory disease complex (Rossow et al., 1995) or vaccination failure (Amadori and Razzuoli, 2014; Li and Yang, 2003; Suradhat et al., 2006; Zhou et al., 2012). The findings indicated that PRRSV infection could affect overall immunocompetency of the infected pigs. Recently, the emergence of the variant PRRSV strain, known as a highly pathogenic (HP)-PRRSV, has been reported (Zhou et al., 2011). The clinical manifestation of HP-PRRSV is different from that of the typical PRRSV strains, causing severe clinical outcomes and high mortality rate among the infected pigs (Chen et al., 2014; Gao et al., 2015). In contrast to the typical PRRSV, infection of HP-PRRSV resulted in strong pro-inflammatory cytokine production in the infected host (Amarilla et al., 2015; Liu et al., 2010). However, the underlying mechanisms of the differences in the pathogenicity of the two viruses are not fully understood.

PRRSV primarily infects monocytes/macrophages and dendritic cells, resulting in inhibited expression of MHC and co-stimulatory molecules in the infected cells (Flores-Mendoza et al., 2008; Van Gorp et al., 2008; Wang et al., 2007). Moreover, PRRSV infection promotes apoptosis of the infected cells (Miller and Fox, 2004; Rodriguez-Gomez et al., 2013). In the early phase of infection, PRRSV induces delayed and weak pro-inflammatory cytokines, including IL-1, IL-6 and TNF-α and type I IFN productions (Calzada-Nova et al., 2011; Patel et al., 2010; van Gucht et al., 2003; van Reeth and Nauwynck, 2000). It has also been reported that PRRSV suppressed the production of IL-1β, IL-8 and IFN-α in serum during the first 14 days post-infection (p.i.) (Lunney et al., 2010). In the infected lungs, decreased IL-1, IL-6 and TNF-α production was also observed during the first 10 days of PRRSV infection (van Gucht et al., 2003). Some studies suggested that the non-structural proteins and nucleocapsid protein of PRRSV could modulate type I IFN production *in vitro* (Beura et al., 2010; Sagong and Lee, 2011; Wang and Zhang, 2014). These findings clearly indicated that PRRSV infection significantly interferes with the innate immune functions. In addition, recent publications indicated that type 2 PRRSV infection could induce IL-10 production and viral-specific regulatory T cells (Treg) both *in vitro* and *in vivo* (Chung and Chae, 2003; Lunney et al., 2016; Silva-Campa et al., 2012; Suradhat and Thanawongnuwech, 2003; Wongyanin et al., 2012), which could lead to the inefficient induction of viral-specific immune responses. Interestingly, some of the type 1 PRRSV strains did not induce IL-10 and Treg production, yet still exhibited clinical and immunological manifestation as do type 2 PRRSV (Gimeno et al., 2011; Silva-Campa et al., 2010). Thus, the immunosuppressive mechanism of PRRSV during the early phase of PRRSV infection remains incompletely understood.

Interestingly, our preliminary microarray analysis demonstrated that type 2 PRRSV-infected monocyte-derived dendritic cells (MoDC) exhibited strong upregulation of interleukin-1 receptor antagonist (IL-1R) gene expression (unpublished observation).

IL-1Ra is an early anti-inflammatory cytokine that controls inflammatory responses during an early stage of immune activation (Arend, 2002). IL-1Ra competitively binds to the interleukin-1 receptor and subsequently blocks the intracellular IL-1 signaling cascade (Arend et al., 1998). IL-1Ra is produced by monocytes, macrophages or dendritic cells, which are known to be PRRSV target cells (Arend et al., 1998; Delputte et al., 2005; Van Gorp et al., 2008). IL-1Ra can modulate the production of IL-1 and TNF-**α** (Marsh et al., 1994) and type I IFN (Ludigs et al., 2012). Therefore, early IL-1Ra production could affect induction of pro-inflammatory and antiviral cytokines during the early phase of PRRSV infection. In this study, we aimed to confirm and investigate the induction of IL-1Ra by type 2 PRRSV strains, including the variant HP-PRRSV, using both *in vitro* and *in vivo* infection models.



3.3 Materials and methods

3.3.1 Viruses and cells

Type 2 PRRSV, strain 01NP1 (Thanawongnuwech et al., 2004), HP-PRRSV, strain 10PL01 (Ayudhya et al., 2012), classical swine fever virus (CSFV), swine influenza virus (SIV); H1N1 (AT/swine/Thailand/CU-PS73/2010) and H3N2 (A/swine/Thailand/CU-CB8.4/2007) were kindly provided by the Chulalongkorn University Veterinary Diagnostic Laboratory (CU-VDL; Bangkok, Thailand). PRRSV was propagated and titrated in the MARC-145 cell line (CU-VDL) at 10^6 $TCID_{50}$ ml^{-1}. CSFV and influenza virus were propagated to 10^6 $TCID_{50}$ ml^{-1} in the SK-6 (CU-VDL) and MDCK cell lines (CU-VDL), respectively. Mock-infected cell lysates were prepared from MARC-145 (for PRRSV), SK-6 (for CSFV) and MDCK (for influenza virus) cell lines. All viruses and mock-infected cell lysates were stored at 80°C until needed.

3.3.2 Antibodies and secondary conjugates

Anti-swine CD3-FITC mAb (BB23-8E6, IgG2b) conjugate and biotinylated anti-swine SWC3 mAb (74-22-15, IgG1) were purchased from SouthernBiotech. Anti-swine sIg (H +L)-PE polyclonal Ab (15H6, IgG1) was purchased from AbSerotec. Anti-swine IL-1Ra mAb (114801, IgG2a) and the isotype controls were purchased from R&D Systems. Streptavidin-APC and goat anti-mouse IgG2a-PE/cy7 were purchased from BioLegend. Anti-PRRSV mAb (SDOW-17, RTI, SD, US) was provided by CU-VDL.

3.3.3 Animals and animal experiments

Eight-week-old, crossbred, PRRSV-seronegative pigs (five pigs per group) were obtained from a commercial farm in Kanchanaburi Province, Thailand. The pigs were randomly grouped and housed throughout the experiment at the animal facility of the Faculty of Veterinary Medicine Kasetsart University, Kumpangsan campus the pigs were vaccinated with 2 ml PBS, 2 ml of a commercial type 2 MLV (Ingelvac PRRS Boehringer Ingelheim Vetmedica) or intranasally inoculated with 2 ml of $10^{4.5}$ $TCID_{50}$ ml^{-1} HP-

PRRSV (10PL01). Heparinized whole blood and serum samples were collected at 0, 2, 4, 7 and 14 days p.i. The blood samples were subjected for PBMC isolation and further immunofluorescent staining. Serum samples were subjected for detection of PRRSV using quantitative reverse transcriptase PCR (see below). The samples were collected from the experimental pigs specifically for the purposes of this study.

3.3.4 Isolation of porcine PBMC and PBL, generation of MoDC and in vitro activation assays

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood samples by gradient centrifugation, using LymphoSep^{TM} separation medium (MP Biomedicals) according to the manufacturer's protocol. For the generation of MoDC, the PBMC were resuspended at $5x10^6$ cells ml^{-1} in Iscove's modified Dulbecco's medium (Gibco) and plated on a 24-well plate (Corning-Costar) for 2h. Non-adherent cells, containing 74.7±8.35 % CD3 and 16.1±3.76 % sIg cells, referred to as peripheral blood lymphocytes (PBL), were collected and stored in liquid nitrogen until needed. The remaining adherent cells, referred to as monocytes, were cultured in 1 ml of RPMI medium containing advanced RPMI (Gibco), 10 % FBS (Gibco), 2 mM L-glutamine (Gibco), antibiotic/antimycotic solution (Gibco), 25 mM HEPES (Gibco) and 50 μM β-mercaptoethanol (Sigma Chemical), supplemented with 10 ng ml^{-1} porcine recombinant IL-4 (R&D Systems) and 25 ng ml^{-1} porcine recombinant GM-CSF (R&D Systems), referred to as DC medium, for 7 days, with 50 % replacement with fresh DC medium every 2 days. For in vitro activation, the porcine leukocyte populations (PBMC, PBL and MoDC) were cultured with 0.1 m.o.i. of the indicated viruses or the relevant mock-infected cell lysates on a 24-well plate at 37°C in a 5 % CO_2 incubator for 24 or 48 h, as indicated in the legends.

3.3.5 Microarray analysis

MoDC were cultured with 0.1 m.o.i. of type 2 PRRSV (01NP1) or MARC-145 cell lysate on a 24-well plate at 37°C in a 5 % CO_2 incubator for 24 h. Total mRNA was purified using a commercial RNA extraction kit (Qiagen) according to the manufacturer's instructions. Samples were hybridized onto Porcine_GXP_4x44K (two-colour microarray-based gene expression analysis). The arrays were scanned with an Agilent scanner system, and data were collected using an Agilent Feature Extraction Software v10.7. Data were normalized using GeneSpring GX 11.5 software. The fold values were provided in terms of log base 2 when comparing the two groups: PRRSV (P) and mock-infected MoDC (M).

All microarray data have been approved and deposited in the Gene Expression Omnibus under accession number GSE86182.

3.3.6 Quantitative reverse transcriptase PCR detection of PRRSV RNA

Viral RNAs were extracted from the sera using an RNA extraction kit (NucleoSpin RNA virus kit; MACHEREY-NAGEL) according to the manufacturer's protocol. Quantification of PRRSV RNA was performed using TaqMan probe-based real-time reverse transcriptase PCR. The sequences of the probe and primers are as follows: US-PRRSV-specific probe (5' FAM-TCC-CGG-TCC-CTT-GCC-TCT-GGA-TAMRA 3'), ORF7-US forward primer (5' AAA-TGI-GGC-TTC-TCI-GGI-TTTT 3') and ORF7-US reverse primer (5' AAA-TGI- GGC-TTC-TCIG-GIT-TTT 3'). The amplification was carried out in a 25 µl reaction containing SuperScript III One-Step RT-PCR kit (Thermo Fisher Scientific) 1x reaction mix, 0.4 mM of each primer and probe, 0.5 µl (100 U) of SuperScript III RT/Platinum Taq Mix and 0.5 µl (20 *p*mol) of viral RNA.

3.3.7 qPCR for detection of porcine cytokine gene expression

Total mRNAs were extracted from the leukocyte subpopulations (PBMC, PBL and MoDC) using a commercial RNA extraction kit (Biotechrabbit, Germany) according to the manufacturer's instructions. The extracted mRNAs were quantified using NanoDrop (Thermo Scientific) and converted to cDNA using a commercial cDNA synthesis kit (Invitrogen). The levels of cytokine gene expression were determined using SYBR Green-based real-time PCR. The qPCR for detection of porcine *IL1RA*, *IL1B*, *IFNA*, *IL10* and *GAPDH* gene expression was performed using the primer sets described in Table 3.1. The qPCR was carried out in a 20 µl reaction and consisted of 2 µl (20 ng) of the cDNA template, 0.5 µl (20 pmol) of each specific primer, 7 µl of sterile water and 10 µl of SYBR Green master mix (Biotechrabbit). qPCR was carried out using a Rotor-Gene RG-3000 (Corbett Research). The amplification reaction consisted of initial denaturation at 95°C for 15 min, followed by 40 cycles of denaturation at 94°C for 20 s, annealing at 55°C for 30 s and extension at 72°C for 30 s. The specificity of the amplicon was verified by melting curve analyses. The Ct values of gene expression were normalized against the housekeeping gene GAPDH. Differences in Ct values between the treatment groups were analysed by the formula 2^{-ddCt}.

Table 3.1 oligonucleotide sequences of primer used for qPCR

Gene	accession no.	Primer sequence (5'>3')	Product size (bp)	References
IL1RA	AY577820.1	F: CTG-GAG-GAG-GAC-TCC-AT R: GAG-TCT-GTC-TTG-CAG-GTT	268	-
IL1B	NM_214055.1	F: AAC-GTG-CAA-TGA-TGA-CTT-TG R: CAC-TTC-TCT-CTT-CAA-GTC-CC	292	-
IL6	JQ839263.1	F: AGA-ACT-CAT-TAA-GTA-CAT-CCT-CG R: AGA-TTG-GAA-GCA-TCC-GTC	180	-
IFNA	XM_003480507.3	F: CTG-GAG-GAG-GAC-TCC-AT R: GAG-TCT-GTC-TTG-CAG-GTT	268	-
IL10	HQ236499.1	F: AGC-CAG-CAT-TAA-GTC-TGA-GAA R: CCT-CTC-TTG-GAG-CTT-GCT-AA	394	(Wongyanin et al., 2010)
GAPDH	XM_005658673.2	F: AAG-TGG-ACA-TTG-TCG-CCA-TC R: TCA-CAA-ACA-TGG-GGG-CAT-C	318	(Wongyanin et al., 2010)

3.3.8 Detection of IL-1Ra in the cultured supernatants

Following in vitro activation, supernatants obtained from the cell culture were collected and stored at -80°C until used. Determination of IL-1Ra protein was performed using a commercial ELISA kit (CUSABIO), according to the manufacturer's protocol.

3.3.9 Immunofluorescent staining and flow cytometric analyses

The cells of interest were harvested, washed once with Ca^{2+}, Mg^{2+}-free PBS (PBSA) and then resuspended in PBSA supplemented with 0.5 % BSA and 0.1% sodium azide, referred to as the FACS buffer. The cells were distributed ion a 96-well plate (Corning Costar) at the concentration of $2x10^6$ cells per well and pelleted by centrifugation at 500 g for 5 min. Immunofluorescent staining of the surface molecules, CD3, SWC3 and sIg, was performed by addition of 1:50 of anti- CD3-FITC, 1:100 of biotinylated anti-SWC3 and 1:100 of anti-sIg (H+L)-PE antibodies, diluted in FACS buffer and followed by incubation at 4°C in the dark for 40 min. The stained cells were then

washed twice with PBSA. For secondary staining of SWC3, 1:500 of streptavidin-APC diluted in FACS buffer was added to the cells followed by incubation at 4°C in the dark for 30 min.

For intracellular staining, the protein transport inhibitor monensin (GolgiStop; BD Biosciences), was added to the culture 12 h prior to harvesting. Following harvesting and staining of the surface molecules, the cells were then fixed and permeabilized with 100 µl per well of 50% reagent A (Leucoperm, Serotec), then diluted in FACS buffer at room temperature in the dark for 30 min. For primary staining, 1:100 of anti-IL-1Ra (IgG2a), diluted in reagent B (Leucoperm), was added to the cells and further incubated at 4°C in the dark for 45 min. For the secondary staining required for staining of IL-1Ra, 1:100 of goat anti-mouse IgG2a-PE/cy7, diluted in FACS buffer, was added to the cells followed by incubation at 4°C in the dark for 30 min. As a final step, the stained cells were resuspended in 2 % formaldehyde in PBSA and kept at 4°C until flow cytometric analyses were performed.

Similar cells stained with isotype controls were included and used as the background cut-off in this study. Fluorescent minus one staining samples were also performed during the establishment and validation of the assay. Flow cytometric analyses were performed using a Beckman Coulter FC 500 MPL.

3.3.10 Statistical analyses

Data were analysed using student t-test or ANOVA, followed by Tukey's multiple comparison tests all statistical analyses were performed using GraphPad Prism for Windows (GraphPad Software Incorporated).

3.4 Results

3.4.1 PRRSV upregulated *IL1RA* gene expression in cultured porcine MoDC and PBMC.

To investigate the factors involved in virus-induced immunomodulation during the early phase of PRRSV infection, porcine MoDC were cultured in the presence of type 2 PRRSV (01NP1) for 24 h, and the total RNA was subjected to microarray analysis using the Porcine_GXP_4x44K (Two-colour microarray-based gene expression platform). The relative expression of the innate, T-helper polarizing and inhibitory cytokine genes is shown in Fig. 3.1A (upper panels). Strong upregulation of genes *IL10* and *IL1RA* was clearly evident. Upregulations of genes *IFNA*, *IFNB*, *IL1B* and *IL2* were also observed. The *in vitro* findings on upregulation of type I IFN gene were consistent with previous reports (Lee et al., 2004; Nan et al., 2012). Interestingly, while most of the type I IFN receptor downstream signaling genes were upregulated, consistent with the enhanced type I IFN gene expression, upregulation of the IL-1 receptor downstream signaling genes was not evident in the system (Fig. 3.1A, lower panels). The results indicated that the presence of PRRSV upregulated expression of *IL1RA* and *IL1B*, but not the IL-1 receptor downstream signaling genes, in the cultured MODC.

To confirm the above findings, the levels of *IL1RA* gene expression in the MoDC cultured in the presence of type 2 PRRSV (01NP1) or other swine respiratory viruses were analysed using quantitative real-time PCR (qPCR). Classical swine fever virus (CSFV) and swine influenza virus, which had been reported to induce strong pro-inflammatory cytokines (IL-1, IL-6 and TNF-α) and type I IFN production during the early phase of infection (Bensaude et al., 2004; Sladkova and Kostolansky, 2006; Van Reeth, 2000), were also included in the experiment. The levels of *IL1RA* gene expression determined by qPCR are shown in Fig. 3.1B. The results demonstrated that the presence of PRRSV, but not other swine viruses, significantly enhanced *IL1RA* gene

expression in the cultured MoDC. In addition, it should be pointed out that PRRSV-induced *IL1RA* gene expression was much stronger than that from the cells treated with lipopolysaccharide (LPS) (Fig. 3.1B). The kinetics of PRRSV- induced *IL1RA* gene expression in the cultured porcine PBMC were investigated and are shown in Fig. 3.1C. The percentages of PRRSV-positive cells in the myeloid subpopulation (SWC3 cells) after 3, 6, 12 and 24 h incubation were 12.2±1.6%, 14.4±3.2%, 24±2.6% and 30.1±2.9%, respectively (data not shown). Upregulation of the *IL1RA* gene was evident from 3 h and peaked at 12 h following infection with type 2 PRRSV (Fig. 3.1C). Interestingly, the level of *IL1RA* gene expression in the PRRSV-infected PBMC remained higher than that from LPS treatment at 24 h (Fig. 3.1C). The data suggested that the presence of PRRSV enhanced and prolonged *IL1RA* gene expression in the culture system.

To confirm the gene expression experiments, the levels of IL-1Ra protein in the cultured supernatants were determined by ELISA. Consistent with the aforementioned results, the presence of PRRSV enhanced IL-1Ra production in the cultured MoDC (Fig. 3.2A). The levels of IL-1Ra protein detected in the supernatants correlated well with those of *IL1RA* gene expression (r^2=0.7664, P<0.001) (Fig. 3.2B).

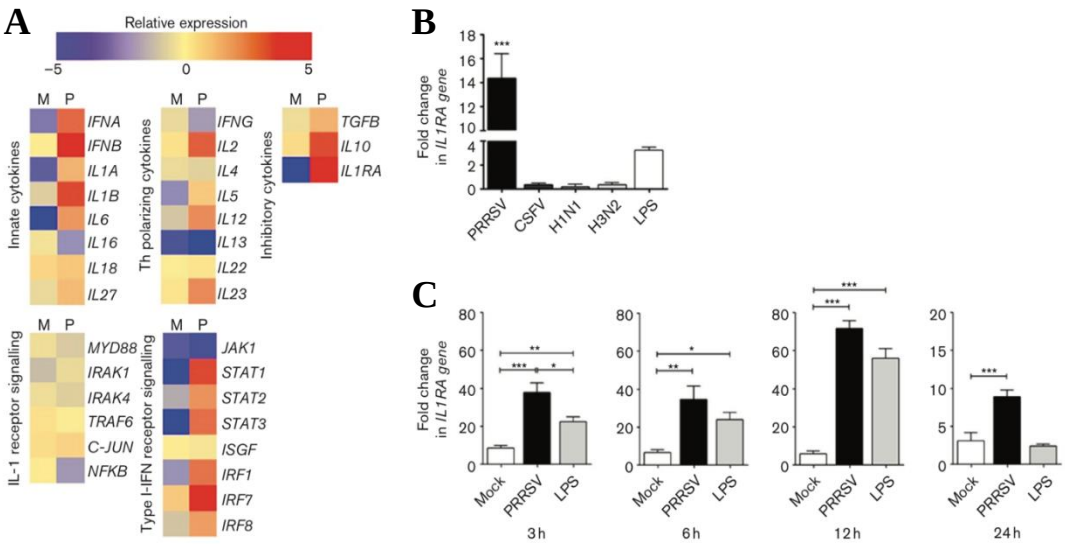


Figure 3.1 PRRSV upregulated *IL1RA* gene expression in the cultured porcine leukocytes. (A) Microarray analysis of cytokine genes and related cytokine signaling genes between the MODC treated with type 2 PRRSV (P) and mock-infected MoDC (M) was compared. Porcine MoDC were cultured in the presence of 0.1 m.o.i. of type 2 PRRSV (01NP1) or MARC-145 cell lysate for 24 h. (B) PRRSV significantly upregulated *IL1RA* gene expression. Porcine MoDC (five pigs per group) were cultured with type 2 PRRSV (01NP1), classical swine fever virus (CSFV), H1N1, H3N2, lipopolysaccharide (LPS) or the relevant mock-infected cell lysate for 24 h. Data represent the fold changes in the levels of *IL1RA* gene expression obtained from the treatment subtracted by those of the relevant mock-infected cell lysate. (C) Kinetics of *IL1RA* gene expression in the PRRSV- and mock-infected PBMC were performed at 3, 6, 12 and 24 h. PBMC were cultured with 0.1 m.o.i. of type 2 PRRSV (01NP1) or MARC-145 cell lysate or 0.1 μg ml^{-1} LPS (five pigs per group), then harvested at the indicated time point. ***, ** and * indicate significant difference at $P<0.0001$, $P<0.001$ and $P<0.05$, respectively.

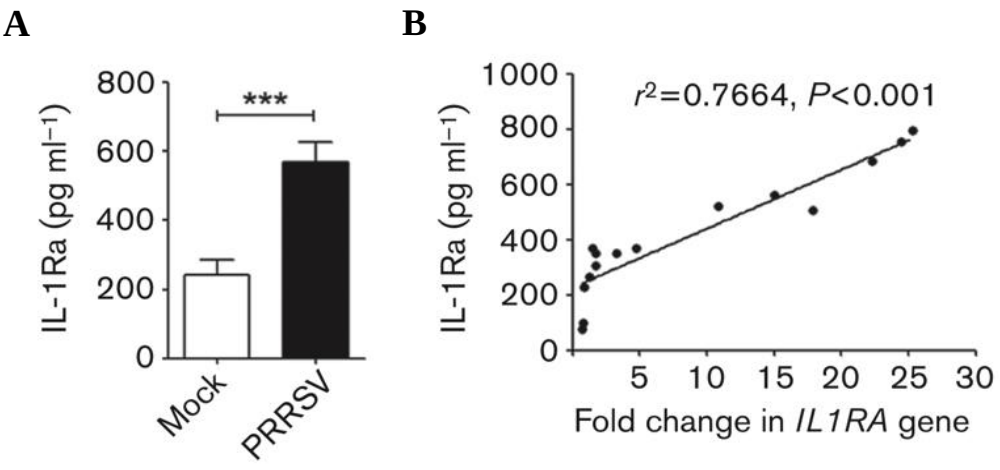


Figure 3.2 PRRSV-induced IL-1Ra production correlated well with the levels of *IL1RA* gene expression. (A) Levels of secretory IL-1Ra in the MoDC cultured supernatants. (B) Correlation of the levels of IL-1Ra detected by ELISA and those of *IL1RA* gene expression. Porcine MoDC (eight pigs per group) were cultured with 0.1 m.o.i. of type 2 PRRSV (01NP1) or MARC-145 cell lysate for 24 h. *** indicates significant difference at $P<0.0001$.

3.4.2 The myeloid population was responsible for IL-1Ra production in the PRRSV infection model

To determine the porcine leukocyte subpopulation responsible for IL-1Ra production in this model, various porcine leukocyte subpopulations were cultured *in vitro* with type 2 PRRSV for 24 h. The presence of type 2 PRRSV resulted in significant upregulation of *IL1RA* gene expression in the cultured MoDC and PBMC, but not in the peripheral blood lymphocyte (PBL) subpopulation (Fig. 3.1A). The IL-1Ra protein production by porcine leukocytes was further confirmed by flow cytometry. The cultured PBMC were initially gated into myeloid and lymphocyte subpopulations based on the forward and side scatter profiles. The cells were further identified by the expression of SWC3 (myeloid), CD3 (T lymphocyte) and surface immunoglobulin (sIg) (B lymphocyte) markers. The identified lymphocyte subpopulations consisted of the cells that highly expressed CD3 (T lymphocytes) and sIg (B lymphocytes), while the myeloid subpopulation primarily expressed the SWC3, but not CD3 and sIg, marker (Fig. 3.1B). The numbers of myeloid cells obtained from the cells treated with PRRSV and mock- infected PBMC were insignificant (data not shown). The results indicated that the presence of type 2 PRRSV significantly enhanced IL-1Ra production in the myeloid, but not lymphocyte subpopulations (Fig. 3.1C, D). Together, the results indicated that porcine myeloid population is the major IL-1Ra producer in this culture system, and the presence of PRRSV significantly enhanced IL-1Ra production from this cellular population.

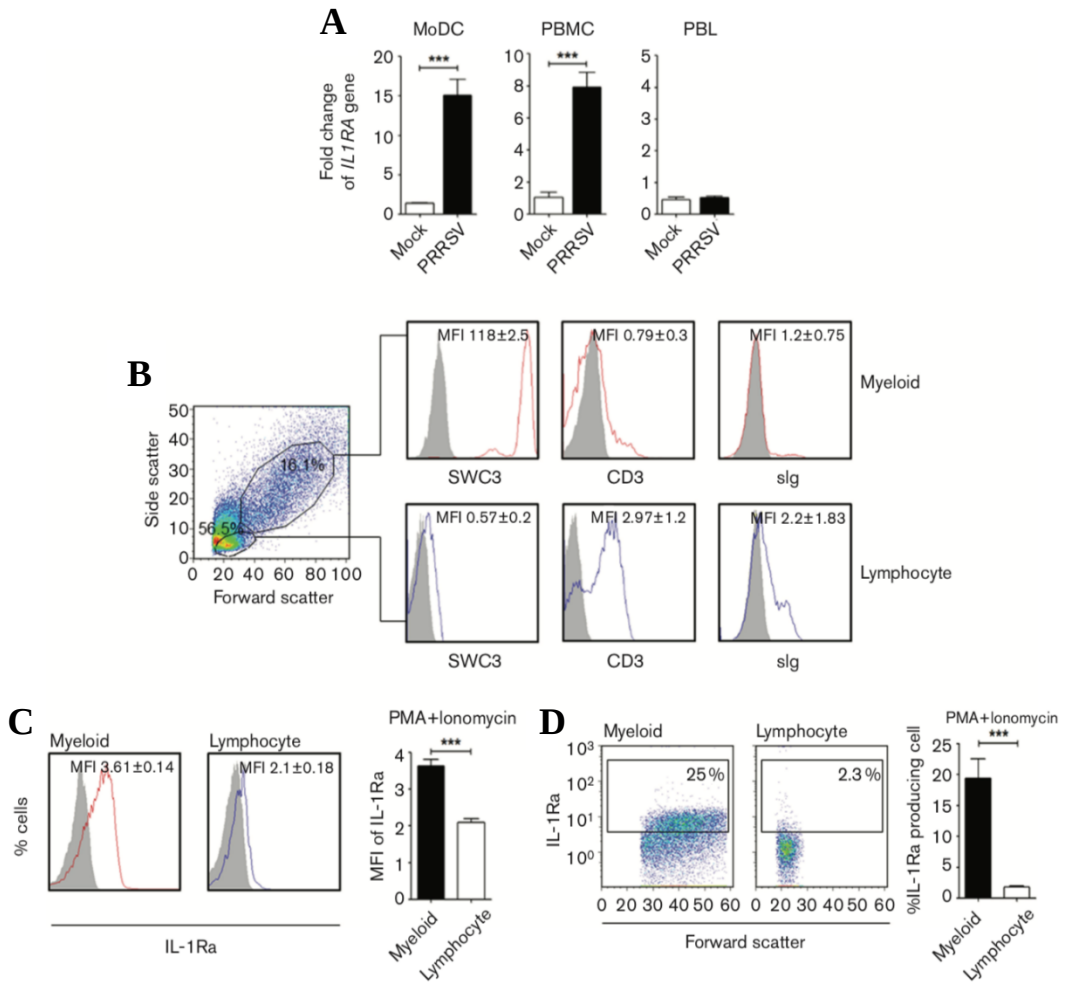


Figure 3.3 PRRSV enhanced IL-1Ra production in the myeloid subpopulation *in vitro*. (A) PRRSV upregulated *IL1RA* gene expression in the cultured MoDC and PBMC, but not PBL. (B) Characteristics of porcine myeloid and lymphocyte subpopulations. Porcine leukocyte subpopulations were identified based on the expression of the surface markers; SWC3 (myeloid cells), CD3 (T lymphocytes) and sIg (B lymphocytes). (C) Mean fluorescent intensity (MFI) of IL-1Ra production and (D) Percentages of IL-1Ra-producing cells in the indicated leukocyte subpopulations. Porcine PBMC (five pigs/group) were cultured with 0.1 m.o.i. of type 2 PRRSV (01NP1) or MARC-145 cell lysate for 48 h. Data represent the MFI or percentage of IL-1Ra-producing cells obtained from sample treated with PRRSV subtracted by that of mock-infected PBMC. *** indicates significantly different at $P<0.0001$.

3.4.3 PRRSV infection increased numbers of IL-1Ra producing cells in infected pigs.

To confirm the induction of IL-1Ra by PRRSV in vivo, PRRSV-seronegative pigs were vaccinated with the commercially available type 2 modified-live PRRS vaccine (MLV), which has previously been shown to possess immunomodulatory properties similar to the field strain PRRSV (Charerntantanakul, 2012; LeRoith et al., 2011). On days 0, 4, 7 and 14 post-vaccination (p.v.), PBMC were collected from the immunized pigs to assess the numbers of IL-1Ra-producing cells by flow cytometry. The myeloid and lymphocyte subpopulations were gated and identified, based on the expression of SWC3, CD3 and sIg surface markers (see above) (Fig. 3.4A). The percentages of the myeloid subpopulation obtained from the MLV and PBS control groups were comparable (data not shown). Consistent with the *in vitro* findings, immunization with type 2 PRRS-MLV significantly enhanced IL-1Ra production in the myeloid subpopulation (Fig. 3.4B, C). Viral genomic RNA was detected from 4 days p.v. and remained high throughout the observation period, confirming ongoing PRRSV infection in the vaccinated pigs (Fig. 3.4D). The kinetics of IL-1Ra production between the experimental groups are shown in Fig. 3.4E. The numbers of IL-1Ra-producing cells in the myeloid subpopulation significantly increased from 4 to 7 days p.v. (Fig. 3.4E). Therefore, consistent with the *in vitro* findings, type 2 PRRSV enhanced IL-1Ra production in the myeloid subpopulation of the infected pigs.

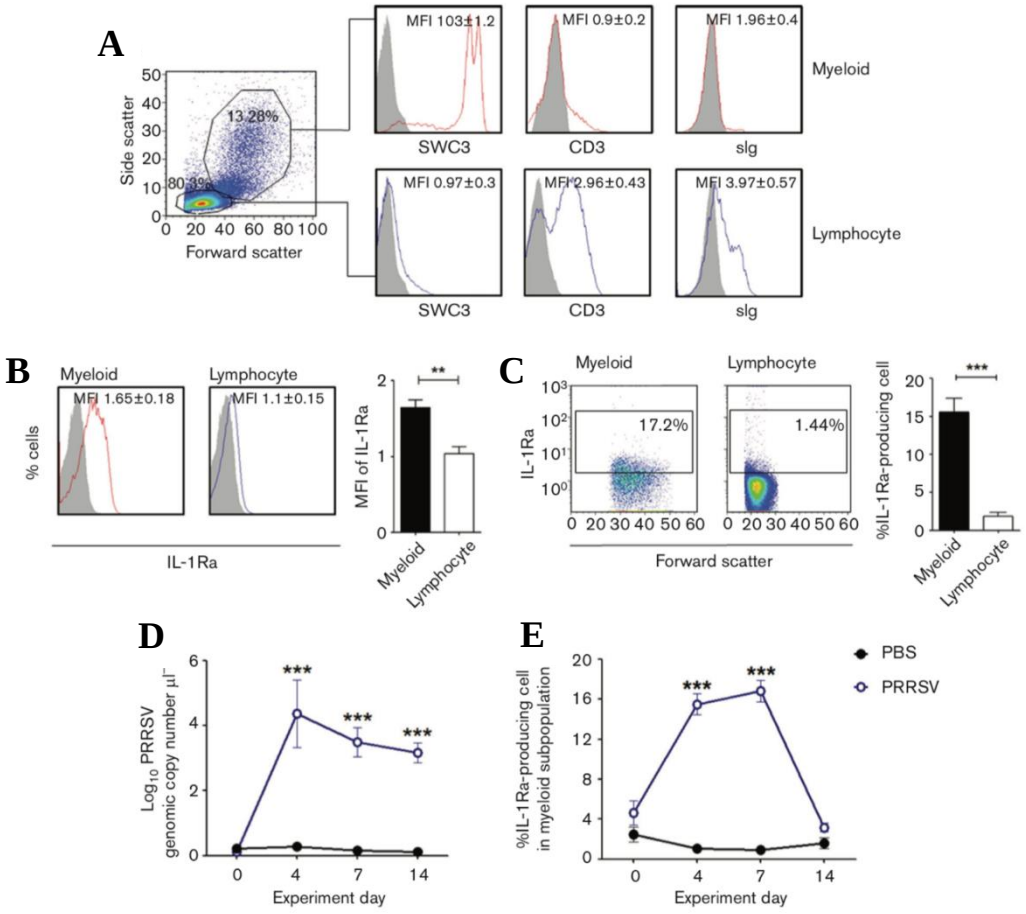


Figure 3.4 PRRSV enhanced IL-1Ra-producing cells in pigs receiving PRRS-MLV. (A) Characteristics of the myeloid and lymphocyte subpopulations in the PBMC of the pigs receiving PRRS-MLV. (B) Mean fluorescent intensity (MFI) and (C) Percentage of IL-1Ra-producing cells in the indicated leukocyte subpopulations. (D) PRRSV genomic copy numbers detected in the sera of the experimental pigs and (E) percentages of IL-1Ra-producing cells in the myeloid subpopulation during the observation period. PRRSV-seronegative pigs (eight pigs per group) were vaccinated with type 2 PRRSV-MLV or PBS at 0 days p.i. Subsequently, serum and heparinized whole blood samples were collected at 0, 4, 7 and 14 days p.i. *** and ** indicate significant difference at $P<0.0001$ and $P<0.001$, respectively.

3.4.4 HP-PRRSV induced different patterns of *IL1RA* and pro-inflammatory cytokine gene expression compared to type 2 PRRSV.

HP-PRRSV exhibits different clinical outcomes and patterns of inflammatory cytokine responses compared with the typical type 2 PRRSV strain (Guo et al., 2013a; Zhang et al., 2013). In this study, the kinetics of *IL1RA* and other innate cytokine gene expression in the presence of type 2 PRRSV (01NP1) and HP-PRRSV (10PL01) were compared. In contrast to type 2 PRRSV, HP- PRRSV did not enhance *IL1RA* gene expression in the cultured MoDC (Fig. 3.5A). To confirm the *in vitro* findings, PRRSV-seronegative pigs were vaccinated with type 2 PRRS-MLV or intranasally inoculated with $10^{4.5}$ $TCID_{50}$ of HP-PRRSV (10PL01). HP-PRRSV (10PL01) infection resulted in high fever, depression, severe respiratory distress and pathological changes, consistent with the clinical manifestation patterns induced by HP-PRRSV (Zhou and Yang, 2010) (data not shown). Viral genomic RNA was detected in the infected pigs from 2 days p.i. to the end of the observation period (14 days p.i.) (Fig. 3.5B). In the pigs receiving MLV, upregulation of *IL1RA* gene expression was clearly observed (Fig. 3.5C), while upregulation of *IL1B* and *IFNA* gene expression was not evident during the same period (Fig. 3.5D, E). Upregulation of *IL10* gene expression was observed later than that of *IL1RA* at 4 days p.v. (Fig. 3.5F). Consistent with the *in vitro* findings, HP-PRRSV infection did not upregulate *IL1RA* gene expression in the infected pigs throughout the experiment (Fig. 3.5C). Infection with HP-PRRSV significantly enhanced *IL1B* and *IFNA* gene expression in the infected pigs, from 2 days p.i. (Fig. 5D, E), while upregulation of *IL10* gene expression was observed from 7 days p.i. Together, our findings indicated that type 2 PRRSV and HP-PRRSV induced different patterns of *IL1RA* and other innate cytokine gene expression.

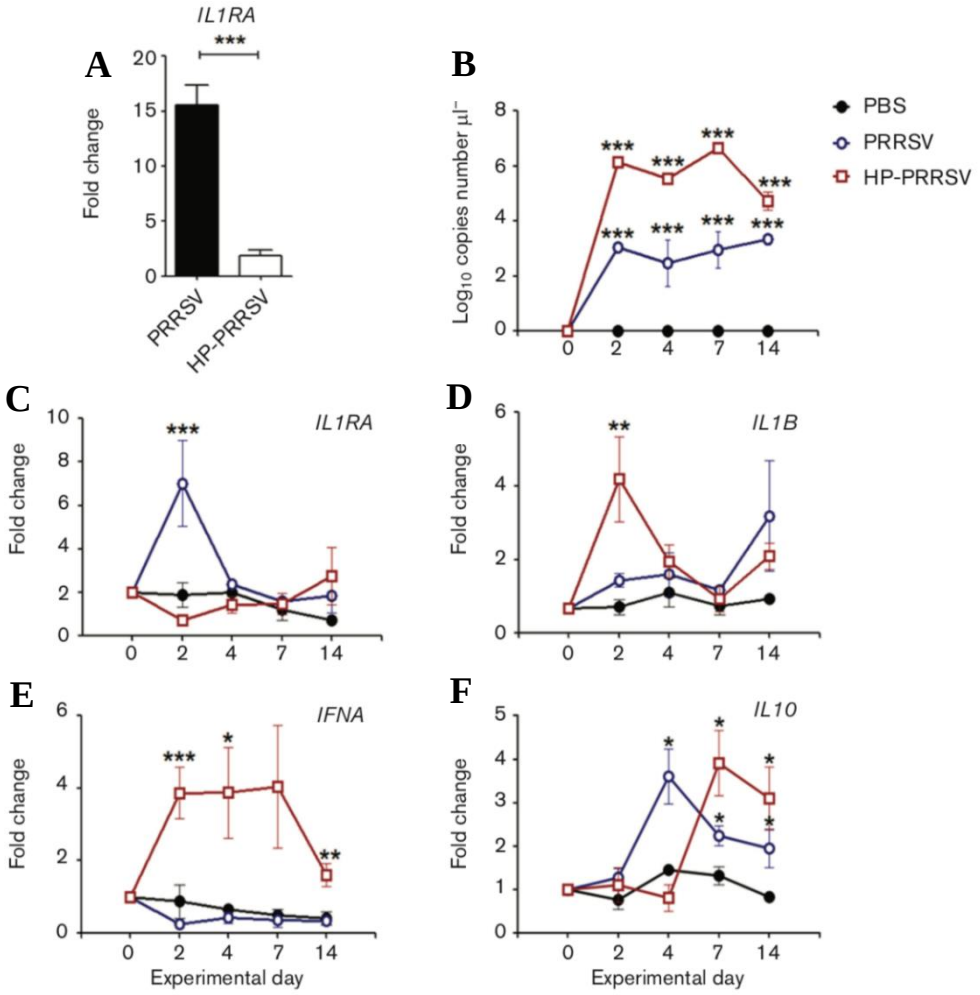


Figure 3.5 HP-PRRSV induced different patterns of *IL1RA* and other cytokine gene expression. (A) Levels of *IL1RA* gene expression induced by PRRSV and HP-PRRSV. Porcine MoDC (five pigs per group) were cultured with 0.1 m.o.i. of HP-PRRSV, 0.1 m.o.i. of type 2 PRRSV (01NP1) or MARC-145 cell lysate for 24 h. Data represent the level of *IL1RA* gene expression obtained from the treatment subtracted by that of mock-infected MoDC. The levels of (B) PRRSV genomic copy numbers, (C) *IL1RA*, (D) *IL1B*, (E) *IFNA* and (F) *IL10* gene expression in pigs of the experimental groups during the observation period. PRRSV-seronegative pigs were vaccinated with type 2 PRRSV-MLV or intranasally inoculated with HP-PRRSV, PBS (five pigs per group) at 0 days p.i. Subsequently, serum and heparinized whole blood samples were collected at 0, 2, 4, 7 and 14 days p.i. ***, ** and * indicate significant difference at $P<0.0001$, $P<0.001$ and $P<0.05$, respectively.

3.5 Discussion

To investigate the immunomodulatory mechanism of early PRRSV infection, we identified IL-1Ra as a potential negative immunomodulatory mediator. The effects of PRRSV on induction of IL-1Ra were demonstrated, both *in vitro* and *in vivo*. Notably, both type 2 PRRSV (strain 01NP1) and type 2 PRRS-MLV were able to induce IL-1Ra production in this study. The findings supported previous opinions, indicating that the immunomodulatory properties of PRRS-MLV are similar to those of field PRRSV (Charerntantanakul, 2012; LeRoith et al., 2011). PRRSV primarily infects the myeloid cell population, including macrophages and dendritic cells (Delputte et al., 2005; Van Gorp et al., 2008), which appear to be responsible for IL-1Ra production. Consistent with the findings in this study, the cells of myeloid lineage, including monocytes/ macrophages, dendritic cells and neutrophils, have been reported as the major IL-1Ra producers in humans (Arend and Guthridge, 2000; Jenkins and Arend, 1993; Jenkins et al., 1994; Re et al., 1993). It should be noted that the production of IL-1Ra was observed at the very early stage following PRRSV infection, concurrent with the absence of pro-inflammatory cytokines and type I IFN gene expression, while enhanced IL-10 gene expression was observed at the later time point (Fig. 3.5). Although it has been indicated that PRRSV-induced IL-10 can negatively modulate the host immune responses (Silva-Campa et al., 2012; Song et al., 2013), the results from this study suggested that PRRSV-induced IL-1Ra production, rather than IL-10, was likely responsible for the decrease in pro-inflammatory cytokine and type I IFN production during the early phase of PRRSV infection. The findings on different patterns of IL-1Ra production following type 2 PRRSV and HP-PRRSV infections further highlighted the potential regulatory role of IL-1Ra, which could affect the clinical outcome following PRRSV infections.

IL-1Ra is known as an early inhibitory cytokine that which plays an important regulatory role in acute inflammatory response (Arend and Guthridge, 2000). One

previous report demonstrated that IL-1Ra could modulate the production of pro-inflammatory cytokines and type I IFN; IL-1Ra suppressed the production of IL-1α-induced IL-6 and IL-8 in the epithelial cells (Banda et al., 2005). Exogenous IL-1Ra inhibited the production of IL-1β and TNF-α in LPS-stimulated monocytes (Ludigs et al., 2012; Marsh et al., 1994). In rheumatoid arthritis patients, treatment with synthetic IL-1Ra peptide drastically decreased the production of IL-1β, IL-6 and TNF-α in the affected joints (Schiff, 2000). In addition, it has been shown that IL-1Ra modulated IFN-α and IFN-β production in a mouse model (Gao et al., 2015; Mayer-Barber et al., 2014). IL-1Ra also modulated the adaptive immune functions by reduction of antigen-specific T-cell activation, proliferation and polarization (Matsuki et al., 2006; Nakae et al., 2003; Wang et al., 2006). Furthermore, lack of an IL-1 receptor signaling cascade inhibited the polarization of T-helper type 2 and production of antigen-specific antibodies (Nakae et al., 2001; Wang et al., 2006). Together, the above information indicates that IL-1Ra production influences host immunocompetency.

In general, typical PRRSV infection induced weak innate cytokine production and delayed cell-mediated and humoral immunity in infected pigs (van Reeth and Nauwynck, 2000). It is likely that early induced and prolonged IL-1Ra production will influence the induction of innate and, subsequently, adaptive immune responses resulting in the unique immunological profiles observed following PRRSV infection. Supporting this notion, enhanced IL-1Ra production played a crucial role in suppression of IL-1/IL-8-induced inflammatory responses during an early phase of Yersinia pestis infection. It is believed that induction of IL-1Ra accommodated bacterial survival within the infected lungs (Sivaraman et al., 2015). The induction of IL-1Ra by Aspergillus fumigatus correlated with increased fungal burden and suppressed inflammation in the infected lungs (Gresnigt et al., 2014). Overexpression of IL-1Ra enhanced Listeria monocytogenes replication and interfered with the macrophage maturation process in

infected mice (Irikura et al., 1999). In HIV-infected patients, levels of serum IL-1Ra correlated with decreased CD8 activation and enhanced viral load during the acute phase of HIV infection (Chevalier et al., 2013). The biological relevance of PRRSV-induced IL-1Ra on the functions of porcine innate and adaptive immune functions is currently under investigation.

In contrast to typical PRRSV, HP-PRRSV induces strong innate cytokine production during the early phase of infection and causes severe clinical disease in infected pigs (Amarilla et al., 2015; Chen et al., 2014; Liu et al., 2010). Our findings that HP-PRRSV enhanced high *IL1B* and *IFNA* gene expression (Fig. 3.5D, E) are consistent with the above reports. To date, the reasons for variation in the pathogenesis of HP-PRRSV are not clearly understood (Do et al., 2015; Guo et al., 2013b). In contrast to the typical type 2 PRRSV, HP-PRRSV did not induce IL-1Ra, either *in vitro* or *in vivo*. Our findings suggest that inability to induce IL-1Ra production could be one of the underlined mechanisms, resulting in uncontrolled production of innate cytokines and severe clinical outcome following HP-PRRSV infection. Interestingly, mice lacking IL-1Ra exhibited enhanced production of pro-inflammatory cytokines, including IL-1, IL-6 and IL- 17, and increased mortality in the subsequent Ebola virus infection (Hill-Batorski et al., 2015). The role of IL-1Ra in the immunopathogenesis of HP-PRRSV should be further explored.

It should be pointed out that different PRRSV strains possess different immunomodulatory properties, in particular, induction of IL-10 production and viral-specific Treg (Rodriguez-Gomez et al., 2015; Silva-Campa et al., 2010; Subramaniam et al., 2011). In this study, the potential of other PRRSV strains, including type 1 (EU) PRRSV, and other type 2 PRRSV strains or other PRRSV-MLV, to induce IL-1Ra production was not explored due to limited availability of the viruses. It would be interesting to further investigate whether the ability to induce IL-1Ra production is conserved among the PRRSV strains. In addition, the precise molecular mechanism responsible for PRRSV-

induced IL-1Ra production is not known. This information will be crucial for better understanding of the immunopathogenesis of PRRSV.

In conclusion, this study identified IL-1Ra as a negative immunomodulatory mediator during the early phase of PRRSV infection. In addition, type 2 PRRSV and HP-PRRSV exhibited variation in their capability in regard to induction of IL-1Ra. These findings could partly explain the differences in immunopathogenesis and clinical outcomes following PRRSV infections.

CHAPTER IV

Negative immunomodulatory effects of porcine reproductive and respiratory syndrome virus-induced interleukin-1 receptor antagonist on porcine immune responses

Manuscript in preparation

Negative immunomodulatory effects of porcine reproductive and respiratory syndrome virus-induced interleukin-1 receptor antagonist on porcine immune responses

Teerawut Nedumpun, Navapon Techakriengkrai, Roongroje Thanawongnuwech and Sanipa Suradhat

PRRSV infection interferes with host immune functions, which subsequently lead to delayed anti-viral immune responses. Induction of IL-1Ra has been previously proposed as one of PRRSV-induced immunosuppressive strategies during the early phase of infection. Here, we addressed the effects of PRRSV-induced IL-1Ra on porcine immune functions and confirmed the effects of PRRSV-induced IL-1Ra on the inhibition of several innate immune functions, T lymphocyte proliferation and differentiation, and induction of regulatory T lymphocytes. Our findings confirm the role of IL-1Ra as a key negative immunomodulator and help paving toward better understanding of the immunopathogenesis of PRRSV infection.

4.1 Abstract

Impaired innate and adaptive immune responses are evidenced through the course of PRRSV infection. We previously reported that PRRSV-induced production of interleukin-1 receptor antagonist (IL-1Ra), an inhibitory cytokine, involved in immunomodulation during an early phase of PRRSV infection. However, the exact mechanism(s) related to how PRRSV-induced IL-1Ra could modulate the host immune responses remains limited. To explore the immunoinhibitory roles of PRRSV-induced IL-1Ra on porcine immune responses, monocyte-derived dendritic cells (MoDC) and leukocytes were cultured with type 2 PRRSV, and IL-1Ra neutralization was performed by addition of anti-porcine IL-1Ra mAb. The results demonstrated that PRRSV-induced IL-Ra was responsible for inhibition of phagocytosis, expressions of MHC II (SLA-DR) and CD86 molecules, as well as down regulation of *IFNA* and *IL1* gene expressions in the culture system. Interestingly, PRRSV-induced IL-1Ra obtained from the infected MoDC also inhibited T lymphocyte proliferation and differentiation, but not IFN-γ production. Although PRRSV-induced IL1-Ra was not directly linked to IL-10 production, it contributed to the differentiation of regulatory T lymphocytes (Treg) within the culture system. Taken together, our results demonstrated that PRRSV-induced IL-1Ra interferes with innate immune functions, T lymphocyte differentiation and proliferation, and acts collectively with IL-10 in Treg induction. The roles of IL-1Ra elucidated in this study help completing the mechanism of PRRSV immunopathogenesis and may eventually lead to better disease intervention.

Keywords: PRRSV, IL-1Ra, neutralization, porcine immune responses

4.2 Introduction

Porcine respiratory and reproductive syndrome virus (PRRSV) is one of the major pathogens affecting pig production industry worldwide. PRRSV is an enveloped, positive-stranded RNA virus, which belongs to the genus *Rodartevirus*, family *Arteriviridae* of the order *Nidovirales* (Kuhn et al., 2016). PRRSV can be divided into two genotypes; type 1 (EU) and type 2 (US) (Brar et al., 2014). However, rapid evolutionary rate often leads to emergence of variant strains of PRRSV (Goldberg et al., 2003; Rowland et al., 1999). Most PRRSV strains are capable of interfering with host immunity, leading to generalized immunosuppression in infected pigs (Kimman et al., 2009). As a result, PRRSV infection usually increases the severity of other concomitantly infected pathogens (Jung et al., 2009).

Innate immunity is recognized as a key modulator for induction of efficient anti-viral immune responses (Iwasaki and Medzhitov, 2015; Iwasaki and Pillai, 2014). However, previous studies provided strong evidences showing that PRRSV primarily infects innate immune cells, including macrophages and dendritic cells (DC), and suppresses their functions (Ke and Yoo, 2017; Sang et al., 2011). It was reported that PRRSV could inhibit maturation of antigen presenting cells (APC) and decrease levels of both MHC II (SLA-DR) and co-stimulatory molecule (CD80 and CD86) expressions (Park et al., 2008; Rodriguez-Gomez et al., 2013). Moreover, enhanced expression of program cell death-ligand 1 (PD-L1) on PRRSV-infected APC was also associated with induction of cell apoptosis and regulatory T lymphocytes (Treg) differentiation (Richmond et al., 2015). During an early phase of PRRSV infection, productions of type I IFN and pro-inflammatory cytokine (IL-1, IL-6 and TNF-α) were drastically suppressed, resulting in uncontrollable viral replication (van Reeth and Nauwynck, 2000; Wang and Zhang, 2014). It has been suggested that PRRSV suppression of innate immunity potentially causes poor adaptive immune responses (Lunney et al., 2016), as

evidenced by attenuated T lymphocyte proliferation and delayed induction of PRRSV-specific IFN-γ-producing cells (Butler et al., 2014; Meier et al., 2003). In addition, weak and delayed neutralizing antibodies responses was often observed in PRRSV infected pigs (Lopez and Osorio, 2004).

The negative immunomodulatory mechanism induced by type 2 PRRSV has always been linked to interleukin-10 (IL-10) induction, which in turn provides immunological niche for Treg expansion (Lunney et al., 2016; Wongyanin et al., 2012). However, the immunosuppressive effect of PRSSV might not be solely attributable to the induction of IL-10 and Treg as these factors appear rather slowly, approximately a week after the suppression of innate immunity in the early phase of PRRSV infection (Chung and Chae, 2003; Wongyanin et al., 2010). Moreover, levels of IL-10 and Treg induction were different among the PRRSV strains (Charerntantanakul et al., 2006).

Recently, it has been proposed that interleukin-1 receptor antagonist (IL-1Ra) induced by PRRSV, might play an important role during the early phase of PRRSV infection. In that study, increased level of IL-1Ra was observed in both *in vitro* and PRRSV-infected pigs (Nedumpun et al., 2017). IL-1Ra competitively binds to IL-1 receptor (IL-1Ra), and subsequently inhibits IL-1-induced signaling cascades (Arend et al., 1998). APC maturation and induction of type I IFN, IL-1 and TNF-α productions are greatly modulated by IL-1Ra production (Arend et al., 1998; Marsh et al., 1994; Sims and Smith, 2010). Moreover, blocking of IL-1R signaling pathway is associated with subversions of antigen-specific T lymphocyte activation and proliferation (Matsuki et al., 2006; Sims and Smith, 2010). Altogether, these evidences strongly suggest the biological effect of IL-1Ra as a key immunomodulator, acting at the very early phase of immune responses. In this study, the roles of PRRSV-induced IL-1Ra on porcine innate and adaptive immune responses were investigated. Our results demonstrated that PRRSV-induced IL-1Ra interferes with innate immune activation, T lymphocyte differentiation and proliferation, and acts collectively with IL-10 in Treg induction. The

roles of IL-1Ra elucidated in this study help completing the mechanism of PRRSV immunopathogenesis that may eventually lead to better disease intervention.

4.3 Materials and methods

4.3.1 Ethics Statement

The animal care and use protocols for this study were adhered to the Ethical principles and guidelines for the use of animals, National Research Council of Thailand and the Guide for the care and use of laboratory animals, National Research Council, USA. All methods and animal studies were conducted under the approval of Chulalongkorn University Animal Care and Use Committee, Chulalongkorn University (Animal Use Protocol No. 1631029).

4.3.2 Viruses and cells

Type 2 PRRSV strain 01NP1 (Thanawongnuwech et al., 2004) and classical swine fever virus (CSFV) were kindly provided by Chulalongkorn University Veterinary Diagnostic Laboratory (CU-VDL; Bangkok, Thailand). PRRSV and CSFV were cultured and titrated in MARC-145 cell line (CU-VDL) and SK-6 (CU-VDL), respectively. Mock-infected cell lysates were prepared from MARC-145 (for PRRSV) and SK-6 (for CSFV) cell lines. All viruses and mock-infected cell lysates were stored at -80°C until needed.

4.3.3 Antibodies and secondary conjugates

Anti-PRRSV N mAb (SDOW-17, IgG) was purchased from RTI (SD, USA). Anti-porcine IL-1Ra (Q29056, goat IgG) and goat IgG Abs (isotype control) were purchased from R&D system (Minneapolis, MN, USA). Anti-porcine SLA-DR mAb (1053H2-18, IgG2a), anti-porcine CD3-FITC mAb (BB23-8E6, IgG2b), biotinylated anti-porcine CD4 mAb (74-12-4, IgG2b) and anti-porcine CD8-PE mAb (76-2-11, IgG2a) were purchased from SouthernBiotech (Birmingham, AL, USA). Anti-human CD86-PEcy7 mAb (IT2.2, IgG1) was purchased from BioLegend® (San Diego, CA, USA). Biotinylated anti-porcine IFN-γ mAb

(P2C11) was purchased from BD Biosciences (San Jose, CA, USA). Anti-porcine IL-10 mAb (945A4C437B1, IgG1) was purchased from Biosource (Camarillo, CA, USA). Anti-porcine CD25 mAb (K231.3B2, IgG1) was purchased from AbD Serotec (Kidlington, UK). Anti-human FOXP3 mAb-APC (236A/E7, IgG1) was purchased from eBioscience (SanDiego, CA, USA). Anti-BrdU-FITC (3D4, IgG1), streptavidin-APC and streptavidin-PEcy7 were purchased from BioLegend®. Goat anti-mouse IgG1-Alexaflur 647 and streptavidin-PE were purchased from ThermoFisher Scientific (Invitrogen, Carlsbad, CA, USA). Goat anti-mouse IgG1-FITC and goat anti-mouse IgG2a-FITC were purchased from AbD Serotec.

4.3.4 Isolation of porcine peripheral blood mononuclear cells (PBMC), peripheral blood lymphocytes (PBL), generation of monocyte-derived dendritic cells (MoDC) and *in vitro* activation

Crossbred, PRRSV-seronegative pigs were previously immunized with CSFV-modified live vaccine (MLV) (COGLAPEST®, Ceva Santé Animale, Libourne, France) at 8 weeks old. Heparinized whole blood samples were collected from the pigs at 12 weeks old. The PBMC were isolated from the heparinized whole blood by gradient centrifugation, using LymphoSep™ (MP Biomedicals, California, USA) according to the manufacturer's procedure. MoDC were generated as described in elsewhere (Nedumpun et al., 2016). Briefly, the PBMC were resuspended in IMDM medium (GIBCO, Carlsbad, CA, USA), and incubated at 37°C in a 5% CO_2 incubator for 2 h. Non-adherent cells, referred as PBL, were collected and stored in liquid nitrogen until needed. The remaining adherent cells, referred as monocytes, were cultured with 10 ng/ml porcine recombinant IL-4 (R&D system, Minneapolis, MN, USA) and 25 ng/ml porcine recombinant GM-CSF (R&D system) for 7 days.

The MoDC or PBMC were cultured with mock (MARC-145 cell lysate) or 0.1 m.o.i. of type 2 PRRSV at 37°C in a 5% CO_2 incubator for 24 or 48 h according to experiment. Supernatants obtained from the cultured MoDC were collected for further

IL-1Ra neutralization assay.

4.3.5 IL-1Ra neutralization assay

After culture with MoDC or PBMC for 24 h, 10 ng/ml of anti-porcine IL-1Ra mAb was added into the culture for neutralization of IL-1Ra. For the control system, the anti-porcine IL-1Ra mAb was replaced with the isotype control antibody.

In some experiments, the supernatants obtained from mock or PRRSV-infected MoDC were incubated with 10 ng/ml of either anti-porcine IL-1Ra or isotype control mAbs for 2 h. The treated supernatants were subsequently cultured with PBL or PBMC.

4.3.6 Phagocytosis assay and induction of dendritic cells maturation and cytokine productions

The MoDC were incubated with FITC-conjugated *E. coli* (ThermoFisher Scientific) at ratio 1:50 (MoDC : *E. coli*). The cells were incubated in a shaker at 37°C for 10 min, then cold PBS was immediately added to stop phagocytic activity. Maturation and cytokine production of MoDC was induced by addition of 1 µg/ml of LPS (Sigma-Aldrich, St. Loius, USA) for 24 h.

4.3.7 T lymphocyte proliferation assay

The PBMC or PBL, which were cultured with anti-IL-1Ra mAb or isotype control, pretreated-supernatants obtained from mock or PRRSV-infected MoDC, were stimulated with 1 µg/ml PHA (Sigma Chemical Co.), DMSO (Sigma Chemical Co.), 0.1 m.o.i. of CSFV or mock-infected cell lysate (SK-6). Immediately after stimulation, 10 µM of Bromodeoxyuridine (BrdU, BioLegend®) was pulsed into the cultures and incubated at 37°C in a 5% CO_2 incubator for 96 h. The cells were harvested and subjected to immunofluorescent staining and flow cytometric analyses.

4.3.8 Detection of PRRSV N protein in the MoDC and PBMC

The porcine leukocyte populations (PBMC and MoDC) were cultured with mock-infected cell lysate (MARC-145) or 0.1 m.o.i. of type 2 PRRSV at 37°C in a 5% CO_2 incubator for 24 h. The cells were then stained and permeabilized with 1:100 anti-PRRSV N mAb (SDOW-17) diluted in Reagent B (Leucoperm, AbD serotec) in the dark at the 4°C for 30 minutes. Subsequently, 1:100 of goat anti-mouse IgG1-FITC mAb were added and incubated in the dark at 4°C for 30 minutes. The stained cells were subjected to flow cytometric analyses.

4.3.9 Detection of IL-1Ra in the cultured supernatants

The supernatants obtained from the cultured MoDC and PBMC were collected and stored at -80°C until used. Levels of IL-1Ra protein were examined using the commercial ELISA kit (CUSABIO, Wuhan, China), according to the manufacturer's protocol.

4.3.10 Immunofluorescent staining and flow cytometric analyses

Immunofluorescent staining of surface molecules including SLA-DR, CD86, CD3, CD4, CD8 and CD25 was performed by addition of primary mAbs at indicated concentration; 1:100 of anti-SLA-DR (IgG2a), 1:50 of anti-CD86-PEcy7, 1:50 of anti-CD3-FITC, 1:50 of biotinylated anti-CD4, 1:50 of anti-CD8-PE, or 1:100 of anti-CD25 (IgG1) mAbs, diluted in PBSA supplemented with 0.5% BSA and 0.1% sodium azide, referred as the FACS buffer, and incubated in the dark at 4°C for 30 minutes. For secondary staining, 1:500 of streptavidin-PE, 1:500 of streptavidin-PEcy7, 1:100 of goat anti-mouse IgG1-FITC or 1:100 of goat anti-mouse IgG2a-FITC, diluted in FACS buffer was added to the cells and incubated in the dark at 4°C for 30 minutes.

For intracellular staining, the cells were then fixed and permeabilized with 50% reagent A (Leucoperm, Serotec), diluted in FACS buffer, for 30 minutes. For primary staining, 1:100 of anti-BrdU-FITC, 1:100 of biotinylated anti-IFN-γ, 1:100 of IL-10 (IgG1)

or 1:20 of anti-FOXP3-APC mAbs, diluted in Reagent B (Leucoperm, Serotec) was added to the cells and further incubated in the dark at 4°C for 45 minutes. For secondary staining, 1:500 of streptavidin-APC or 1:100 of goat anti-mouse IgG1-Alexaflur 647, diluted in FACS buffer was added to the cells and incubated in the dark at 4°C for 30 minutes.

The similar cells stained with isotype controls were included and, used as the background cut-off in the study. The fluorescent minus one (FMO) staining samples were also performed during the establishment and validation of the assay. Flow cytometric analyses were performed using FC 500 MPL (Beckman Coulter, CA, USA).

4.3.11 Quantitative polymerase chain reaction (qPCR)

Total mRNAs were extracted from the cells by using the commercial RNA extraction kit (Biotechrabbit, Germany) according to the manufacturer's instruction. The extracted mRNAs were quantified using NanoDrop (Thermo scientific, USA) and converted to cDNA using a commercial cDNA synthesis kit (Invitrogen, USA). Level of porcine *IFNA, IL1, IL6, TBET, GATA3, RORGT, FOXP3* and *GAPDH* expression was quantified by SYBR green-based qPCR using the primer sets as shown in Table 4.1. qPCR reaction was carried out as previously described (Kiros et al., 2011; Mounsey et al., 2015; Nedumpun et al., 2017; Obremski, 2014). Ct values of each gene were normalized against the housekeeping gene; *GAPDH*. Differences in Ct values between the treatment groups were analyzed by the formula 2^{-ddCt}.

Table 4.1 Sequences of the qPCR primers

Gene	Accession no.	Primer sequence (5' > 3')	Product size (bp)	References
IFNA	XM_003480507.3	F: CTG-GAG-GAG-GAC-TCC-AT R: GAG-TCT-GTC-TTG-CAG-GTT	268	(Nedumpun et al., 2017)
IL1B	NM_214055.1	F: AAC-GTG-CAA-TGA-TGA-CTT-TG R: CAC-TTC-TCT-CTT-CAA-GTC-CC	292	(Nedumpun et al., 2017)
IL6	JQ839263.1	F: AGA-ACT-CAT-TAA-GTA-CAT-CCT-CG R: AGA-TTG-GAA-GCA-TCC-GTC	180	(Nedumpun et al., 2016)
TBET	XM_003132081.4	F: TCA-ATC-CTA-CTG-CCC-ACT-AC R: TTA-GGA-GAC-TCT-GGG-TGA-AC	151	(Obremski, 2014)
GATA3	XM_022745494.1	F: ACA-GAC-CCC-TGA-CCA-TGA-AG R: GGA-GAT-GTG-GCT-GAG-AGA-GG	193	(Obremski, 2014)
RORGT	XM_021089853.1	F: TTC-AGT-ACG-TGG-TGG-AGT-TC R: TGT-GGT-TGT-CAG-CGT-TGT-AG	141	(Mounsey et al., 2015)
FOXP3	XM_021079539.1	F: CTC-CTA-CTC-CCT-GCT-GGC-AAA-T R: TAC-AAT-ACA-GCA-GGA-ACC-CTT-GTC-A	283	(Wongyanin et al., 2010)
GAPDH	XM_005658673.2	F: AAG-TGG-ACA-TTG-TCG-CCA-TC R: TCA-CAA-ACA-TGG-GGG-CAT-C	318	(Wongyanin et al., 2010)

4.3.12 Statistical analyses

Data was analyzed using student *t*-test or analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. All statistical analyses were performed using GraphPad Prism for Windows (GraphPad Software Incorporated, San Diego, CA, USA).

4.4 Results

4.4.1 PRRSV-induced IL-1Ra interfered with phagocytic activity, maturation and production of innate cytokines.

To investigate the role of PRRSV-induced IL-1Ra on porcine innate immune functions, MoDC were first infected with type 2 PRRSV and subsequently cultured with anti-IL-1Ra-specific mAb. Presences of anti-IL-1Ra and isotype control mAbs had no effect on the numbers of PRRSV infected cells (Fig. 4.1A). Prior to addition of the mAbs, the production of IL-1Ra in the culture was quantified, and significant IL-1Ra production was observed in the supernatants of PRRSV-infected MoDC (Fig. 4.1B). PRRSV-infected MoDC exhibited significantly decreased phagocytic activity, which could be restored by the neutralization of IL-1Ra (Fig. 4.1C and D). Next, we investigated the effect of PRRSV-induced IL-1Ra on MoDC maturation. Levels of SLA-DR and CD86 expressions on LPS-induced MoDC were significantly decreased by PRRSV infection (Fig. 4.1E-H), consistent with the previous findings (Chang et al., 2008; Gimeno et al., 2011; Pineyro et al., 2016). Similar to the phagocytic activity, effect of PRRSV infection on SLA-DR (MHC II) and CD86 expression could be mitigated by addition of anti-IL-1Ra (Fig. 4.1E-H).

As downregulation of *IFNA*, *IL1* and *IL6* expression was observed very early, even before the induction of IL-10 during the course of PRRSV infection (Lopez and Osorio, 2004; Van Gucht et al., 2004; Wang and Zhang, 2014; Zhou et al., 2012), we asked whether these effects were related to PRRSV-induced IL-1Ra. As shown in figure 4.2A and B, neutralization of IL-1Ra with anti-IL-1Ra mAb could restore *IFNA* and *IL1* gene expressions up to the similar levels observed in the control treatments (mock). However, neutralization of IL-1Ra appeared to have little effect on the level of *IL6* gene expression (Fig. 4.2C), suggesting the role of unidentified immunomodulatory mediator, in addition to IL-1Ra in the culture system. Altogether, these findings indicated that PRRSV-induced IL-1Ra interfered with functions of APC including phagocytic activity, antigen processing and presentation and certain pro-inflammatory cytokine production.

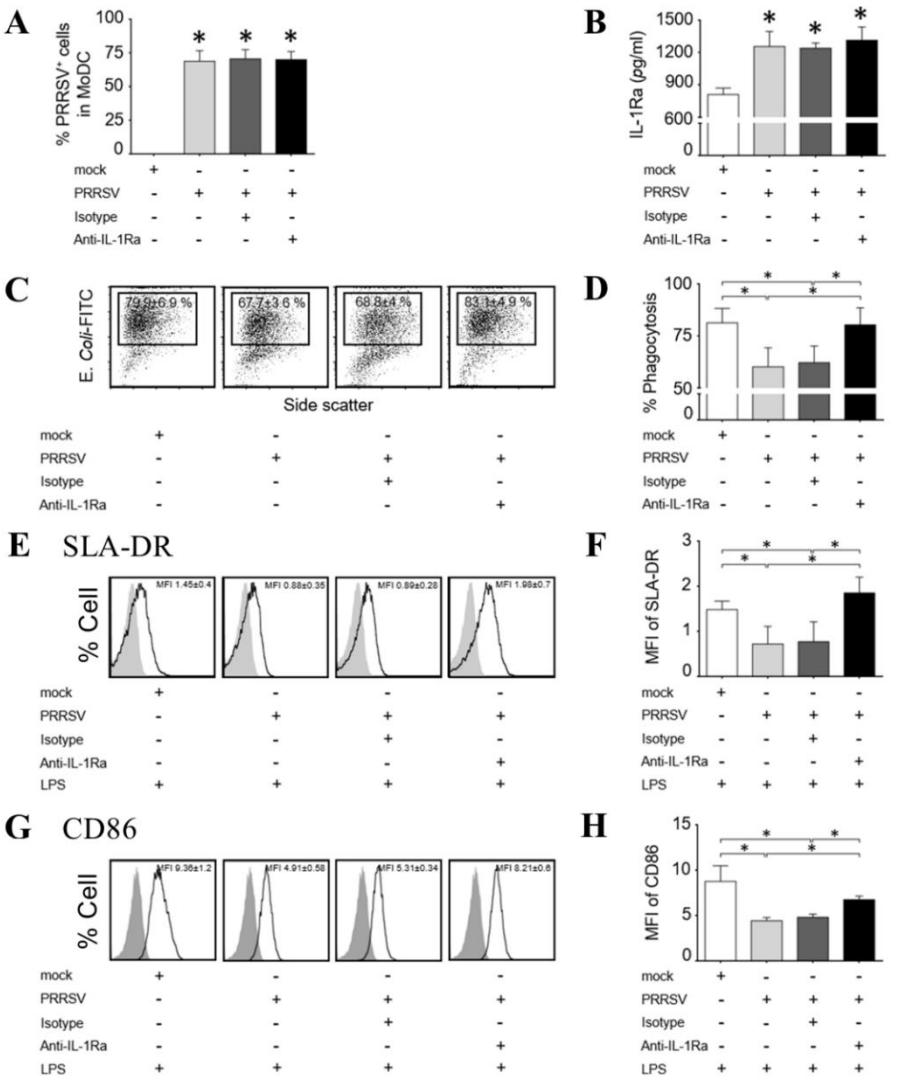


Figure 4.1 PRRSV-induced IL-1Ra interfered with porcine innate immune functions. (A and B) The percentage of PRRSV-infected cells (A) and level of IL-1Ra production (B) in the PRRSV-infected MoDC. * indicates significantly different between control (mock) and other groups at $p<0.05$. (C-H) PRRSV-induced IL-1Ra interfered with phagocytic activity and (C and D) SLA-DR and (E and F) CD86 expressions (G and H). MoDC were cultured with 0.1 m.o.i. of type 2 PRRSV or mock cell lysate (mock) for 24 h. Supernatants from the cultured MoDC were subjected to quantification of IL-1Ra production using ELISA. Anti-IL-1Ra or isotype control mAbs was added into the culture at 2 h post-inoculation. LPS was utilized for induction of MoDC maturation. Histogram presents mean fluorescent intensity (MFI) of SLA-DR or CD86 expression. Data are representative mean of 5 pigs. * indicates significantly different at $p<0.05$.

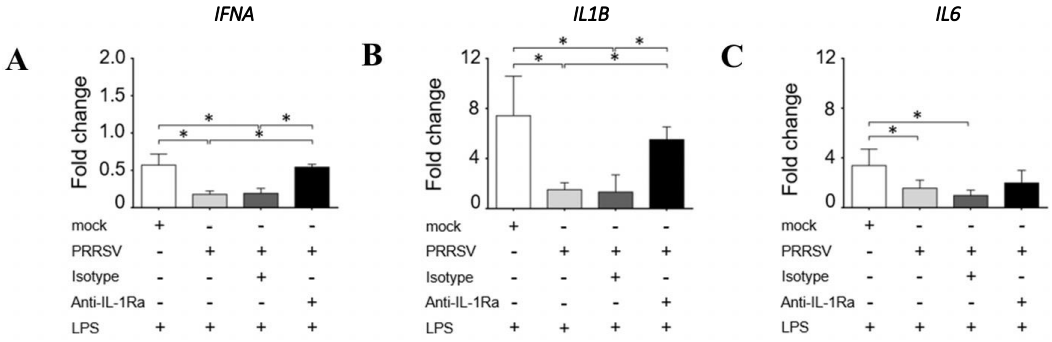


Figure 4.2 Innate cytokine gene expression was modulated by PRRSV-induced IL-1Ra. (A-C) Expressions of *IFNA* (A) and *IL1* (B), but not *IL6* (C) were suppressed by PRRSV-induced IL-1Ra. MoDC were cultured with 0.1 m.o.i. of type 2 PRRSV or mock cell lysate (mock) for 24 h, with or without anti-IL-1Ra mAb. LPS was added for the induction of innate cytokine gene expression. Data are representative mean of 5 pigs. * indicates significantly different at $p<0.05$.

4.4.2 PRRSV-induced IL-1Ra altered CSFV-mediated T helper lymphocyte differentiation.

Differentiations of porcine specific T lymphocyte lineage, namely Th1, Th2, Th17 and Treg, are specifically rendered by constitutive expression of transcription factor (TF); T-bet, GATA3, RORγt and FOXP3, respectively (Kiros et al., 2011). It was indicated in the previous finding that PRRSV co-infection interfered with host specific immune responses against other viral infections including, classical swine fever (CSFV) (Li and Yang, 2003; Rahe and Murtaugh, 2017; Suradhat et al., 2006). Therefore, we determined the immunomodulatory effect of PRRSV-induced IL-1Ra on CSFV-mediated T helper (Th) lymphocyte differentiation, by measuring the levels of the above TF gene expression. Consistent with previous findings (Franzoni et al., 2013; Summerfield and McCullough, 2009; Summerfield and Ruggli, 2015), the presence of CSFV in the culture resulted in strong upregulation of *TBET* expression, which indicates a shift toward Th1 differentiation, upon recalled antigen exposure (Fig. 4.3A). Addition of the supernatant from PRRSV-infected MODC significantly decreased levels of CSFV-induced *TBET* and *GATA3* as compared with supernatant from the control mock cell lysate (Fig. 4.3B and C). This suppressive effects of PRRSV-infected MoDC supernatant on *TBET* and *GATA3* gene expression could be blocked by addition of anti-IL-1Ra mAb (Fig. 4.3B and C). Moreover, addition of anti-IL-1Ra mAb increased the expressions of *TBET*, *GATA3* and *RORGT* to a level even higher than those observed with CSFV alone (Fig. 4.3B-D). As shown in figure 4.3A, CSFV had no effect on *FOXP3* expression. As expected, addition of supernatant from PRRSV-infected MODC to the CSFV-cultured cells drastically upregulated transcriptional *FOXP3* at 48 and 72 h. However, neutralization of IL-1Ra had no effect on the level of *FOXP3* expression (Fig. 4.3E), suggesting the roles of other undefined mechanism(s) on Treg differentiation in the system. Altogether, these results demonstrated the negative effects of PRRSV-induced IL-1Ra on the T cell differentiation upon the recalled antigen, CSFV, exposure.

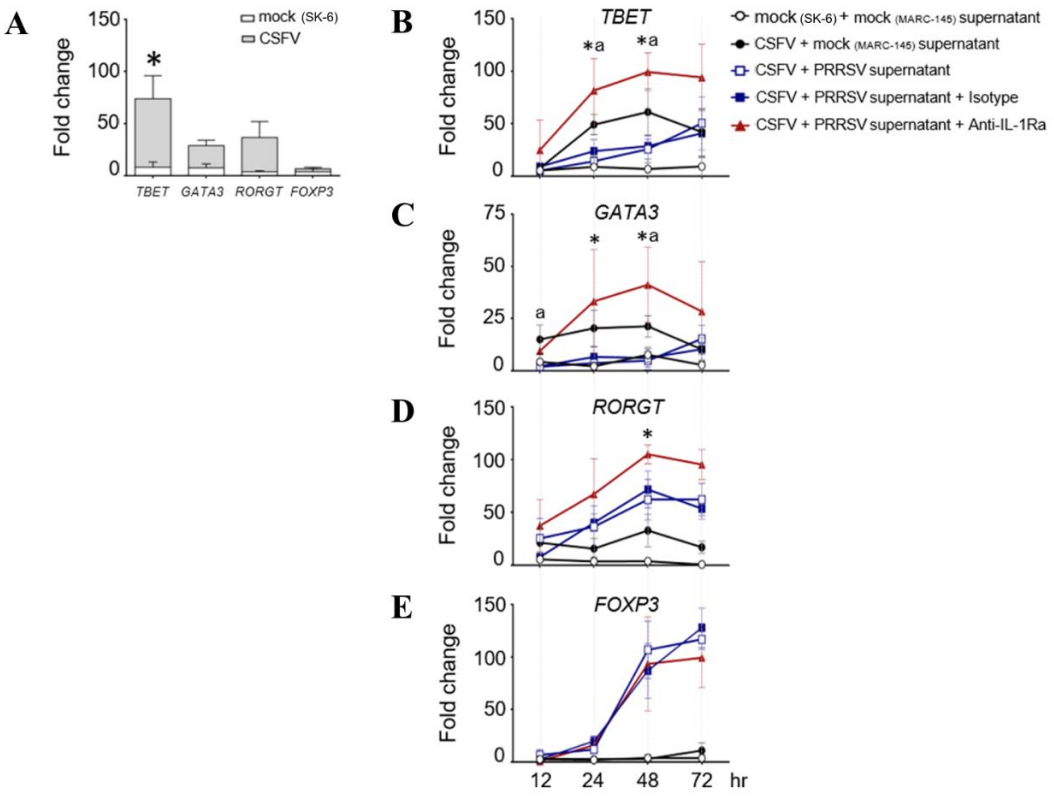


Figure 4.3 PRRSV-induced IL-1Ra possessed a potential subversive effect on CSFV-mediated T helper cell differentiation. (A) CSFV induced strong upregulation of *TBET* in the cultured PBMC at 48 h. * indicates significantly different levels of Th transcription factors (TF) at $p<0.05$. (B-E) Expression of *TBET* (B) and *GATA3* (C), but not *RORGT* (D) were regulated by the PRRSV-induced IL-1Ra. (E) Presence of PRRSV-infected MoDC supernatant strongly induced *FOXP3* expression. MoDC were cultured with 0.1 m.o.i. of type 2 PRRSV or mock (MARC-145 cell lysate) for 24 h. Then, supernatants from the cultured MoDC were pre-treated with anti-IL-1Ra or isotype control mAbs for 2 h. PBMC obtained from CSFV-immuninized pigs were pulsed with 0.1 m.o.i. of CSFV or mock (SK-6 cell lysate), in the presence of pre-treated supernatants. Data are representative mean of 5 pigs at different time points. [a] indicates significantly different between mock (MARC-145 cell lysate)-infected MoDC supernatant (close circle) and PRRSV-infected MoDC supernatant (open and close square) at $p<0.05$. * indicates significantly different between anti-IL-1Ra mAb-treated PRRSV-infected MoDC supernatant (close triangle) and PRRSV-infected MoDC supernatant (open and close square) at $p<0.05$.

4.4.3 PRRSV-induced IL-1Ra participated in inhibition of lymphocytes proliferation, but not effector function.

To investigate the effect of PRRSV-induced IL-1Ra on T lymphocyte proliferation, the supernatants obtained from PRRSV-infected MoDC were pre-treated with anti-IL-1Ra, and added into PHA- or CSFV-stimulated, BrdU-stained porcine PBL or PBMC. Lymphocytes were further gated into $CD4^+CD8^+$ (putative memory T lymphocytes), $CD4^+$ (putative T helper lymphocytes) and $CD8^+$ (putative cytotoxic T lymphocytes) subpopulations (Fig. 4.4A and E). As shown in figure 4.4, the supernatants from PRRSV-infected MoDC significantly reduced the numbers of proliferating $CD4^+CD8^+$, $CD4^+$, and $CD8^+$ in the PHA-activated cultures (Fig. 4.4A-D) and these effects could be prevented by the addition of anti-IL-1Ra mAb. This result indicated that PRRSV-induced IL-1Ra involved in suppression of mitogen-induced lymphocyte proliferation.

We further examined the effect of PRRSV-induced IL-1Ra on antigen-specific T cell proliferation. Consistent with the above finding, proliferations of CSFV-specific $CD4^+CD8^+$, $CD4^+$, and $CD8^+$ lymphocyte subpopulations were significantly reduced in the presence of the supernatants obtained from the PRRSV-infected MoDC, whereas addition of anti-IL-1Ra mAb could restore the CSFV-specific lymphocyte proliferation (Fig. 4.4E-H). This finding confirmed the suppressive effect of PRRSV-induced IL-1Ra on antigen-specific lymphocyte proliferations.

We next investigated the role of PRRSV-induced IL-1Ra on T lymphocyte effector function by enumerating the number of IFN-γ-producing T lymphocytes. Addition of supernatant from PRRSV-infected MoDC significantly reduced the numbers of mitogen-activated and CSFV-specific IFN-γ-producing T lymphocytes (Fig. 4.5A and B). Although the number of IFN-γ-producing cells was slightly increased after adding anti-IL-1Ra into mitogen-induced PBMC, it did not reach that of the mock-control

treatment (Fig. 4.5A). Similarly, addition of anti-IL-1Ra did not restore the numbers of CSFV-specific IFN-γ-producing cells in the culture system (Fig. 4.5B). Together, our data demonstrated that PRRSV-induced IL-1Ra played a role in inhibition of polyclonal and viral-specific lymphocyte proliferation, but not the effector, i.e. cytokine production, function.

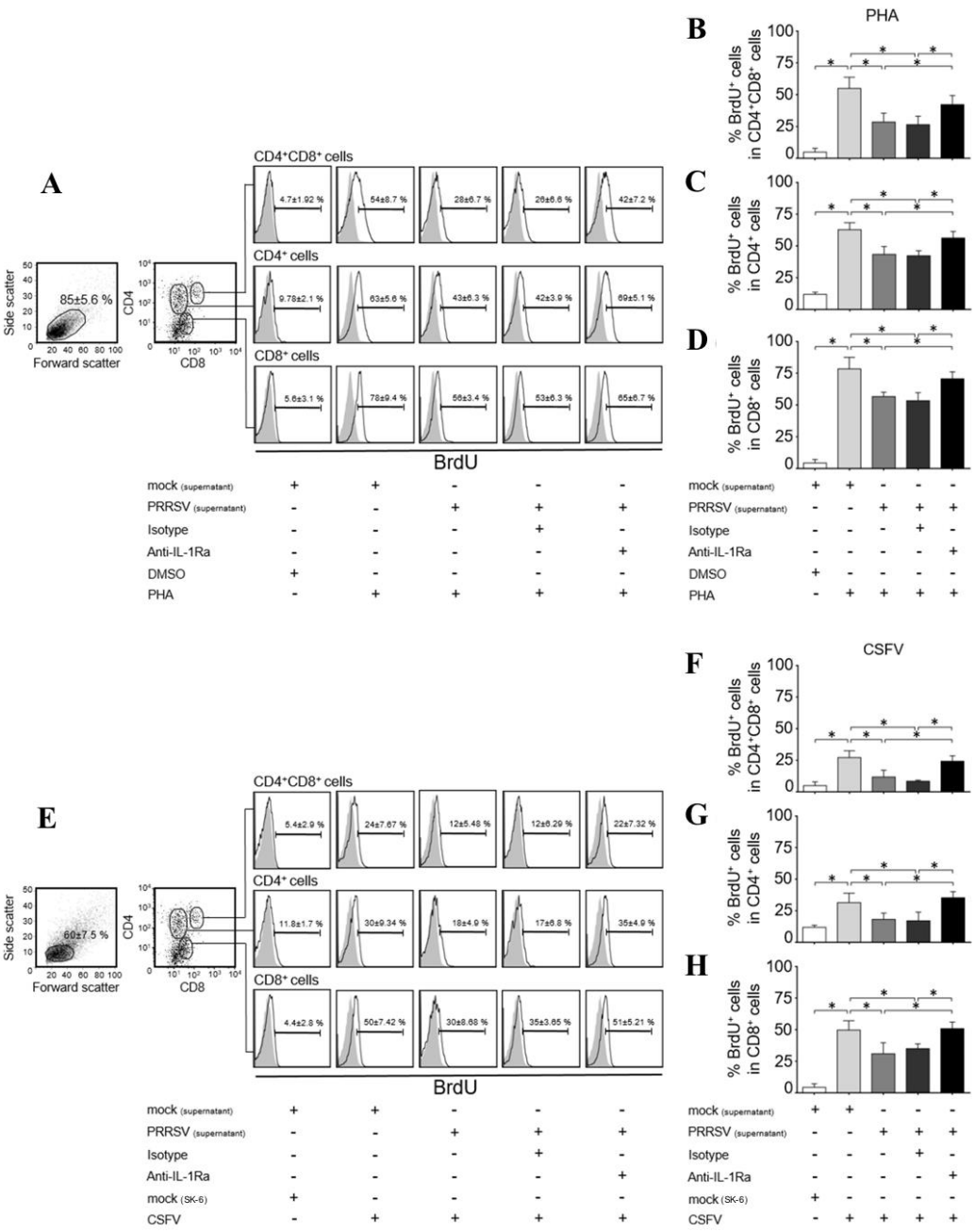


Figure 4.4 IL-1Ra derived by PRRSV-infected MoDC inhibited proliferation of lymphocytes. (A and E) the cells were gated into $CD4^+CD8^+$, $CD4^+$ and $CD8^+$ subpopulations, then further analyzed the proliferative characteristic ($BrdU^+$ cells). (B-D) PRRSV-induced IL-1Ra involved in suppression of non-specific proliferations of $CD4^+CD8^+$ (B), $CD4^+$ (C) and $CD8^+$ (C) subpopulations, as well as (F-H) inhibition of CSFV-specific proliferation of $CD4^+CD8^+$ (F), $CD4^+$ (G) and $CD8^+$ (H) subpopulations. MoDC were

cultured with 0.1 m.o.i. of type 2 PRRSV or mock (MARC-145 cell lysate) for 24 h. Then, supernatants from the cultured MoDC were pre-treated with anti-IL-1Ra or isotype control mAbs for 2 h. PBL or PBMC were pulsed with PHA, 0.1 m.o.i. or CSFV or controls, in the presence of pre-treated supernatants for 96 h. Histogram presents percentage of BrdU positive cells. Data are representative mean of 5 pigs. * indicates significantly different at $p<0.05$.

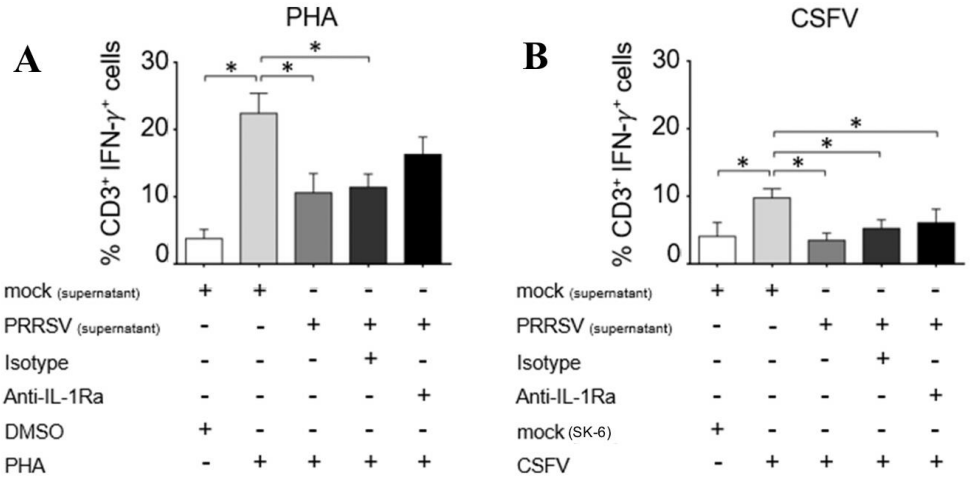


Figure 4.5 IL-1Ra did not involve in PRRSV-suppressed IFN-γ-producing cells in both non-specific (A) **and CSFV-specific conditions** (B). MoDC were cultured with 0.1 m.o.i. of type 2 PRRSV or mock (MARC-145 cell lysate) for 24 h. Then, supernatants from the cultured MoDC were pre-treated with anti-IL-1Ra or isotype control mAbs for 2 h. PBL or PBMC were pulsed with PHA, 0.1 m.o.i of CSFV or controls, in the presence of pre-treated supernatants for 48 h. Data are representative mean of 5 pigs. * indicates significantly different at $p<0.05$.

4.4.4 IL-1Ra partially involved in PRRSV-induced Treg, but not IL-10 production.

As shown earlier, strong upregulation of *FOXP3* was observed in the presence of PRRSV-infected MoDC supernatant. To further investigate the role of PRRSV-induced IL-1Ra on modulation of inhibitory mediators including IL-10 production and Treg induction, PBMC were cultured in the presence of PRRSV, either with or without anti-IL-1Ra mAb. Similar to the MoDC, PRRSV infection significantly enhanced IL-1Ra production in the cultured PBMC (Fig. 4.6A and B). Consistent with previous findings (Chung and Chae, 2003; Song et al., 2013; Suradhat et al., 2003), PRRSV enhanced numbers of IL-10-producing T lymphocytes and Treg (Fig. 4.6C and D). However, addition of anti-IL-1Ra mAb showed little effect on reduction of IL-10-producing cells (Fig. 4.6C). On the contrary, neutralization of IL-1Ra significantly decreased the numbers of Treg in the $CD4^{+}CD25^{+}$ subpopulation, as compared to isotype control or PRRSV alone. However, percentage of Treg was still significantly higher than that of mock control, indicating partial effect of IL-1Ra on induction of Treg in the culture system (Fig. 4.6D). These findings indicated that IL-1Ra partially involved in PRRSV-induced Treg, but not IL-10 production.

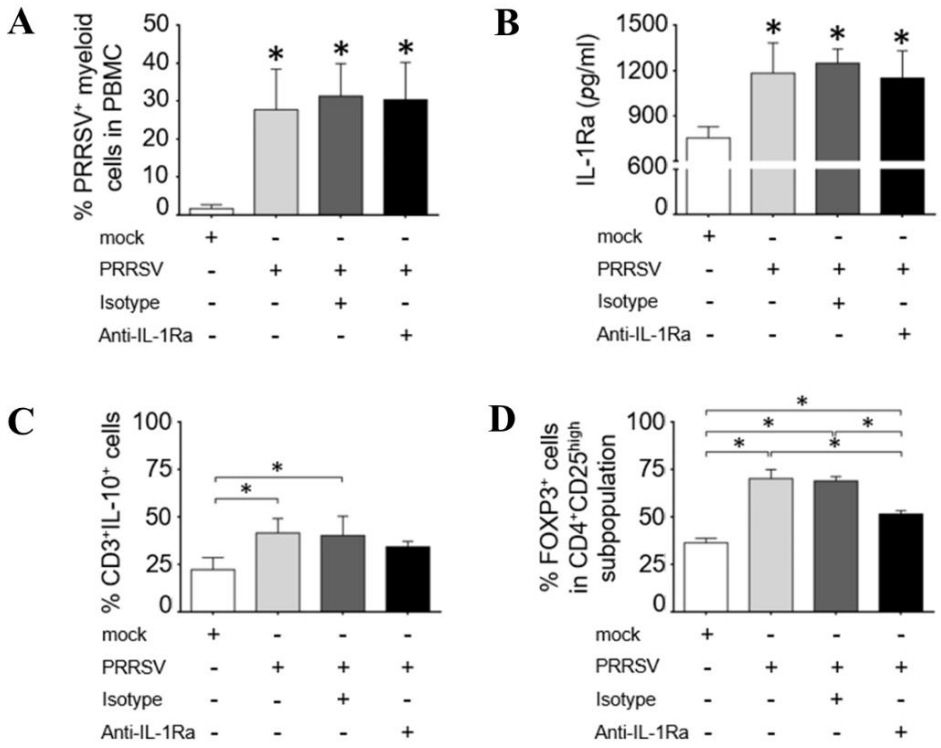


Figure 4.6 PRRSV-induced IL-1Ra participated in enhancing of regulatory T lymphocytes (Treg). (A and B) Percentages of PRRSV-infected cells (A) and levels of IL-1Ra production (B) in the cultured PBMC. (D and C) Addition of anti-IL-1Ra decreased numbers of Treg (D), but not IL-10 producing cells (C). The PBMC were cultured with 0.1 m.o.i. of type 2 PRRSV or mock (MARC-145 cell lysate), with or without anti-IL-1Ra for 48 h. Supernatants from the cultured PBMC were subjected to quantification of IL-1Ra production using ELISA. The cells were stained for flow cytometric analyses. Data are representative mean of 5 pigs. * indicates significantly different at $p<0.05$.

4.5 Discussion

Interleukin-1 receptor antagonist (IL-1Ra) is an early inhibitory cytokine, which potentially participates in PRRSV immunosuppression during the early phase of infection (Nedumpun et al., 2017). Several studies indicated that IL-1Ra acts as a negative immunomodulator for the control of both innate and adaptive immune functions (Ben-Sasson et al., 2009; Granowitz et al., 1992; Iizasa et al., 2005). Here, we demonstrated the negative immunomodulatory effects of PRRSV-induced IL-1Ra on several aspects of porcine immune responses.

In this study, PRRSV-induced IL-1Ra interfered with phagocytic activity and maturation of APC. These findings are consistent with previous studies showing that exogenous IL-1Ra suppressed Fc-dependent phagocytosis (Coopamah et al., 2003), and prevented granuloma formation due to the uncontrollable APC maturation, in IL-1Ra deficient mice (Iizasa et al., 2005). Next, we demonstrated that PRRSV-induced IL-1Ra could reduce *IFNA* and *IL1* gene expressions, which also in agreement with several previous studies reporting the roles of IL-1Ra on type I IFN and pro-inflammatory cytokine production in macrophages and dendritic cells (DC) (Marsh et al., 1994; Mayer-Barber et al., 2014). The immunomodulatory roles of IL-1Ra were also reported in several bacterial infections. Increased level of IL-1Ra production induced by *Yesinia pestis* could suppress pro-inflammatory cytokine productions and prolong bacterial survival during the early stage of infection (Sivaraman et al., 2015). In Leishmanial infection model, lacking of IL-1Ra resulted in massive IL-1 production that lead to severe clinical symptoms (Kautz-Neu et al., 2011). These findings strongly supported the role of IL-1Ra in PRRSV immunopathogenesis. However, our result showed that suppression of *IL6* gene expression by PRRSV was not associated with IL-1Ra production. This finding is similar to a study showing that functional IL-1Ra could not prevent excessive IL-6 production within local injury (Gabay et al., 2001). Hence, it is

very likely that other mechanisms beside IL-1Ra are responsible for PRRSV-suppressed IL-6 production.

PRRSV co-infection has long been shown to cause negative impact on other viral infections, including CSFV (Li and Yang, 2003; Suradhat et al., 2006). In this study, we demonstrated that PRRSV-induced IL-1Ra significantly suppressed lymphocyte proliferation in both antigen-specific and non-specific manners. We also demonstrated that PRRSV-induced IL-1Ra suppressed expressions of *TBET*, *GATA3* and *RORGT* in the CSFV-activated PBMC. The effect of PRRSV-induced IL-1Ra on T lymphocyte proliferation is not unexpected as signaling via IL-1 receptor (IL-1R) was important for induction of T helper lymphocyte (Th) proliferation (Ben-Sasson et al., 2009). Moreover, blocking of IL-1R activation with exogenous IL-1Ra was shown to diminish DNA replication, which resulting in reduction of T lymphocyte expansion (Conti et al., 1991; Rambaldi et al., 1991). IL-1 and IL-1R signaling pathways were also important for inductions of Th1 and Th2 responses (Ben-Sasson et al., 2009). To our surprise, IL-1Ra neutralization resulted in the higher level of *TBET*, *GATA3* and *RORGT* expressions than those from the cells cultured with CSFV alone. This finding suggested that CSFV, and possibly other porcine viruses, could also induce certain levels of IL-1Ra production, similar to what has been observed in HIV-infected patients (Kreuzer et al., 1997; Thea et al., 1996). The virus-induced IL-1Ra production might play an important role in balancing the immune responses as previously reported in several diseases including viral infection (Arend, 2002; Ikeda et al., 2014; Santarlasci et al., 2013). Suppression of TF by PRRSV-induced IL-1Ra most likely contributed to the negative impact of PRRSV on CSFV-specific immune responses, as reported previously (Li and Yang, 2003; Suradhat et al., 2006). Altogether, our finding suggested that the poor adaptive immune response, usually observed in PRRSV infected pigs, is in part due to the blocking of T lymphocyte proliferation and differentiation by PRRSV-induced IL-1Ra.

In addition to proliferation, IFN-γ-production facilitates immunostimulatory effects on phagocytosis, antigen processing and presentation of APC, antiviral stage of the target cells and activation of effector cytotoxic T lymphocytes (CTL) (Schroder et al., 2004). Transcriptomic analysis of PRRSV-immunized pigs revealed that IFN-γ pathway involved in activation of anti-viral defense mechanism (Islam et al., 2017). Here, we demonstrated that PRRSV also suppress IFN-γ production in both mitogen-activated and antigen-specific T lymphocyte responses. Although IL-1R signaling was also reported to play an important role in the T lymphocyte effector and memory functions (Ben-Sasson et al., 2013; Ben-Sasson et al., 2009), neutralization of IL-1Ra could not improve suppression of IFN-γ production. Our finding suggests that other undefined immunomodulatory mechanisms than IL-1Ra were responsible for PRRSV-suppressed IFN-γ production and one of the possible is interleukin-10 (IL-10) as it could interfere with IFN-γ production in T lymphocytes (Ito et al., 1999; Naundorf et al., 2009). In human monocytes and alveolar macrophages, IL-10 could enhance production of IL-1Ra by recruitment of NF-KB to IL-1Ra promotor (Tamassia et al., 2010). Hence, it is possible that suppression of IFN-γ by PRRSV-induced IL-10 help promoting IL-1Ra production instead.

Inductions of IL-10 and Treg are the hallmarks of PRRSV negative immunomodulation (Loving et al., 2015; Silva-Campa et al., 2009; Suradhat et al., 2003). In this study, we found that neutralization of IL-1Ra was ineffective against *FOXP3* gene expression in whole PBMC (Fig. 4.3E) but could partially reduce the percentage of $FOXP3^{+}$ cells in $CD4^{+}CD25^{high}$ subpopulation (Fig. 4.6). This discrepancy might be due to the differences in the culture system and the selected subpopulation. In the PBMC, FOXP3 positive cells are not only composed of T lymphocytes but also macrophages and B lymphocytes, which could also display immunosuppressive activity (Manrique et al., 2011). It is possible that addition of anti-IL-1Ra mAb does not affect *FOXP3*

expression *per se* but blocks the suppressive effect of IL-1Ra on type I IFN production, which in turn help balancing the homeostasis of Treg/Th17 populations, similar to what has been observed in osteoarthritic patients (Lee et al., 2016). It was clearly demonstrated that PRRSV-induced Treg was greatly reduced by neutralization of IL-10 (Wongyanin et al., 2012). Apart from IL-10 production, PRRSV-induced transforming growth factor (TGF)-β, an inhibitory cytokine, involved in differentiation of inducible Treg (Chen and Konkel, 2010; Fan et al., 2015; Fu et al., 2004; Sang et al., 2014; Silva-Campa et al., 2009). PRRSV-induced IL-1Ra might amplify number of inducible Treg by promoting TGF-β as there were some studies reporting that IL-1Ra could enhance TGF-β production in leukocytes and somatic cells (Danis et al., 1995; Yue et al., 1994). Nevertheless, it is obvious that other mechanisms involve in the induction of Treg, in which IL-10 or TGF-β is among the possible mechanisms. Interestingly, our finding indicated that PRRSV-induced IL-1Ra did not associate with IL-10 production in the lymphocyte subpopulation. Regarding this observation, it should be noted that inductions of IL-1Ra and IL-10 by PRRSV do not share the same induction pathway. Previous finding showed that up-regulation of IL-10 production in PRRSV-infected macrophages required NF-KB and p38 MAPK signaling cascades (Song et al., 2013). On the other hands, induction of IL-1Ra in human monocytes was associated with activation of PI3K pathway (Molnarfi et al., 2005). Although information on PRRSV-induced IL-1Ra signaling pathway is not available, it is possible that IL-1Ra and IL-10 production are two distinct mechanisms, collectively participates in PRRSV immunomodulation. These findings convincingly pointed that IL-1Ra also involves in PRRSV-induced Treg differentiation.

Although the immunomodulatory mechanisms of IL-1Ra were thoroughly investigated in this study, there remain several issues to be addressed to completely understand the roles of IL-1Ra in PRRSV immunopathogenesis. Firstly, what is the effect

of PRRSV-induced IL-1Ra on IL-10 production and vice versa in the myeloid cells, as they are major producers of both cytokines (Arend et al., 1998; Song et al., 2013). Is it possible that both cytokines act synergistically to provide for an immunological niche promoting Treg? As high level of IL-1Ra was observed in PRRSV-infected pigs (Nedumpun et al., 2017), whether inhibition of IL-1Ra production in infected animals could provide the same beneficial effects as what observed in our *in vitro* findings remains to be investigated. In addition, the potential for the next generation PRRSV vaccine development, aiming to reduce both IL-1Ra and IL-10 should also be explored.

In conclusion, this study reported the negative immunomodulatory effects of PRRSV-induced IL-1Ra on both innate and adaptive immune functions. Our findings clearly demonstrated suppression of APC functions, including phagocytosis and maturation, and also inhibition of lymphocyte proliferation by PRRSV-induced IL-1Ra. In addition to IL-10, IL-1Ra was also found to participate in PRRSV-induced Treg. This study confirms the role of IL-1Ra as key immunomodulator and help paving toward better understanding of the PRRSV immunopathogenesis and, possibly, better disease intervention.

CHAPTER V

General conclusion, discussion and future recommendations

PRRSV evolves several negative immunomodulatory strategies for evasion of host immune system (Zhang et al., 2012). Poor innate and adaptive immune functions are usually observed throughout the course of PRRSV infection (Loving et al., 2015). *In vitro* MoDC generation system has been extensively used as a tool for studying PRRSV immunomodulation. In chapter II, we demonstrated that modification of MoDC generation protocol could improve the quality of porcine MoDC production system. The MoDC obtained from the modified protocol increased number of $CD1^+$ cells, as well as enhanced phagocytosis and IFN-γ production. The modified protocol was further utilized as a platform for studying interaction of PRRSV and innate immune system in our study, and also had potential for numerous future applications for other disease models.

Several findings indicate that PRRSV markedly interferes with host innate immune functions in an early phase of infection (Calzada-Nova et al., 2011; Patel et al., 2010; van Gucht et al., 2003; van Reeth and Nauwynck, 2000). In chapter III, induction of IL-1Ra by PRRSV had been identified as one of the significant immunosuppressive mechanisms during an early phase of PRRSV infection. IL-1Ra is an early inhibitory cytokine that competitively binds to the interleukin-1 receptor and subsequently blocks the intracellular IL-1 signaling cascade (Arend, 2002; Arend et al., 1998). We confirmed the inductions of IL-1Ra by type 2 PRRSV, both *in vitro* and *in vivo*. In addition, myeloid cells were identified as the major IL-1Ra producer in response to PRRSV infection. In contrast to the type 2 PRRSV, the highly pathogenic (HP) PRRSV did not enhance IL-1Ra production. Our findings suggest that the ability to induce IL-1Ra production in infected hosts could be one of the underlined mechanisms, leading to differences of immunopathogenesis and clinical outcomes following PRRSV infections.

It has been known that different PRRSV strains possess different negative immunomodulatory properties, in particular, induction of IL-10 production and Treg

(Rodriguez-Gomez et al., 2015; Silva-Campa et al., 2010; Subramaniam et al., 2011). In our study, the ability of other PRRSV strains, including type 1 (EU) PRRSV, and other type 2 PRRSV strains or other PRRSV-MLV, to induce IL-1Ra production was not explored due to limited availability of the viruses in our laboratory. It would be interesting to further investigate whether the ability to induce IL-1Ra production is conserved among the PRRSV strains. Although, we reported that HP-PRRSV lacked the ability to induce IL-1Ra production, the roles of IL-1Ra in the immunopathogenesis and disease outcome of HP-PRRSV should be further investigated. In addition, the precise molecular mechanism responsible for PRRSV-induced IL-1Ra production remains unknown. This information will be crucial for better understanding of the immunopathogenesis of PRRSV.

IL-1Ra has been recognized as a key immunomodulatory cytokine, acting at the very early phase of immune responses (Arend et al., 1998). However, the immunomodulatory properties of IL-1Ra involved in regulation of both innate and adaptive immune responses (Arend and Guthridge, 2000). In chapter IV, the findings highlighted the immunoregulatory roles of PRRSV-induced IL-1Ra on porcine innate immune functions and T lymphocyte responses. We demonstrated that PRRSV-induced IL-1Ra interfered with phagocytic activity, maturation of APC and innate cytokine productions. Furthermore, PRRSV-induced IL-1Ra significantly suppressed T lymphocyte differentiation and proliferation. The result also pointed out that PRRSV-induced IL-1Ra was not directly linked to IL-10 production but it involved in the Treg induction. The negative immunomodulatory roles of PRRSV-induced IL-1Ra elucidated in our study help completing the understanding in immunopathogenesis of PRRSV.

Although the immunomodulatory effects of IL-1Ra were thoroughly investigated in our study, there remain several issues to be addressed to completely understand the roles of IL-1Ra in PRRSV immunopathogenesis. Previous findings reported that IL-10 and TGF-β production involved in differentiation of PRRSV-specific Treg (Silva-Campa et al., 2009; Wongyanin et al., 2012). Is it possible that IL-1Ra, IL-10 and TGF-β act synergistically to provide for an immunological niche promoting Treg? As high level of IL-1Ra was observed in PRRSV-infected pigs (Nedumpun et al., 2017), whether inhibition of IL-1Ra production in infected animals could provide the same

beneficial effects as what observed in our *in vitro* findings remains to be investigated. In addition, the potential for the next generation PRRSV vaccine development, aiming to reduce IL-1Ra, IL-10, TGF-β and Treg should also be explored.

In conclusion, the modified MoDC generation system provided better quality of porcine MoDC that had advantage of studying immunological interaction between pathogens and porcine innate immune system in the future. Our finding clearly demonstrated that PRRSV could induce IL-1Ra production, both *in vitro* and *in vivo*. Moreover, we confirmed the negative immunomodulatory effects of PRRSV-induced IL-1Ra on porcine innate and adaptive immune functions. Altogether, our findings help paving toward the better understanding of the PRRSV immunopathogenesis and, possibly, better disease intervention.

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APPENDIX

Appendix A

Generation of potent porcine monocyte-derived dendritic cells (MoDCs) by modified culture protocol

Teerawut Nedumpun,[1] Patcharee Ritprajak[2,3,*] and Sanipa Suradhat[4,5]

[1] Interdisciplinary Program of Medical Microbiology, Graduate School, Chulalongkorn University, Bangkok 10330, Thailand

[2] Department of Microbiology, Faculty of Dentistry, Chulalongkorn University, Bangkok 10330, Thailand

[3] Oral Biology Research Center, Faculty of Dentistry, Chulalongkorn University, Pathumwan, Bangkok 10330, Thailand

[4] Center of Excellence in Emerging Infectious Diseases in Animals, Chulalongkorn University (CU-EIDAs), Bangkok 10330, Thailand

[5] Department of Veterinary Microbiology, Faculty of Veterinary Science, Chulalongkorn University, Bangkok 10330, Thailand.

* Corresponding author:

Assistant Professor Patcharee Ritprajak

Mailing address: RU in Oral Microbiology and Immunology

Department of Microbiology, Faculty of Dentistry,

Chulalongkorn University, Bangkok, 10330, Thailand.

E-mail address: Patcharee.R@chula.ac.th (P. Ritprajak)

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Short communication

Generation of potent porcine monocyte-derived dendritic cells (MoDCs) by modified culture protocol

Teerawut Nedumpun[a], Patcharee Ritprajak[b,c,*], Sanipa Suradhat[d,e]

[a] Inter-department of Medical Microbiology, Graduate School, Chulalongkorn University, Pathumwan, Bangkok 10330, Thailand
[b] RU in Oral Microbiology and Immunology, Department of Microbiology, Faculty of Dentistry, Chulalongkorn University, Pathumwan, Bangkok 10330, Thailand
[c] Oral Biology Research Center, Faculty of Dentistry, Chulalongkorn University, Pathumwan, Bangkok 10330, Thailand
[d] Department of Veterinary Microbiology, Faculty of Veterinary Science, Chulalongkorn University, Pathumwan, Bangkok 10330, Thailand
[e] Center of Excellence in Emerging Infectious Diseases in Animals, Chulalongkorn University (CU-EIDAs), Pathumwan, Bangkok 10330, Thailand

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In vitro culture

ABSTRACT

In vitro derivation of dendritic cells (DCs) is an alternative approach to overcome the low frequency of primary DCs and the difficulty of isolation techniques for studying DC immunobiology. To date, the conventional culture protocol of porcine monocyte-derived DCs (MoDCs) has been widely used. However, this protocol is not practical due to the requirement of a substantial number of blood monocytes, and the process often interferes with DC maturation. To improve *in vitro* porcine MoDC generation, we modified the previous conventional DC generation protocol, based on the human and mouse primary DC culture system, and compared phenotypic and functional features of MoDCs derived from the modified protocol to the conventional protocol. The modified protocol consumed fewer monocytes but generated higher $CD1^+$ cells with DC-like morphology and the ability of maturation. In addition, MoDCs from the modified protocol exhibited increased antigen uptake and IFN-γ production in response to LPS stimulation. Our findings indicate that the modified protocol is expedient and reliable for generating potent MoDCs that substitute for primary DCs. This will be a valuable platform for future research in antigen delivery, vaccines and immunotherapy in pigs, as well as relevant veterinary species.

1. Introduction

Dendritic cells (DCs) are a unique innate immune cell capable of antigen processing and presentation, and they also provide the signal for T lymphocyte activation and differentiation (Merad et al., 2013). Knowledge of the immunomodulatory properties of DCs could lead to improvements in the field of swine vaccinology and immunotherapy. Porcine DCs have been characterized and typified by the expression of the surface molecules, CD1, CD14, CD16, CD80/86, CD172a and MHC class II (Summerfield and McCullough, 2009). However, study of porcine DC biology is limited due to the low frequencies of DC populations in blood circulation and tissues, and laborious DC isolation techniques (Summerfield and McCullough, 2009; Zhang et al., 2009). Therefore, *in vitro* monocyte-derived DCs (MoDCs) is an alternative tool to examine porcine DC biological properties and functions.

Two different *in vitro* culture procedures for porcine DC generation from peripheral blood monocytes have been established. The fast protocol can generate porcine monocyte-derived DCs (MoDCs), so-called fast MoDCs, within 48 h, but the phenotype and function of these DCs are only partially developed (Wongyanin et al., 2012). The conventional protocol requires 7–9 days to induce MoDCs, but these DCs are more potent and favorable for investigating the host- pathogen interaction (Lecours et al., 2011; Summerfield and McCullough, 2009). However, there is a limitation of the conventional protocol, as it requires a large amount of peripheral blood mononuclear cells (PBMCs). In addition, the protocol includes cell harvesting and re-plating steps prior to *in vitro* stimulation which may possibly induce undesirable DC maturation by physical and pressure stimulation (Banchereau et al., 2000). The biological and functional properties of immature and mature DCs are different. Immature DCs are capable of high antigen uptake but low T lymphocyte activation properties, while mature DCs lose their phagocytic ability but can mediate T lymphocyte activation and differentiation (Schnurr et al., 2000). Therefore, the stage of DC maturation is crucial for the downstream experimental interpretation.

The standard protocol for generating *in vitro* DCs in humans and mice are quite similar and have been well validated (Dewitte et al.,

* Corresponding author at: RU in Oral Microbiology and Immunology, Department of Microbiology, Faculty of Dentistry, Chulalongkorn University, Pathumwan, Bangkok 10330, Thailand.
E-mail address: Patcharee.R@chula.ac.th (P. Ritprajak).

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2014; Frindel et al., 1967; Inaba et al., 2009; Qu et al., 2014). Generations of human MoDCs and murine bone marrow-derived DCs (BM-DCs) requires fewer DC precursors than the porcine MoDC protocol. Furthermore, the harvesting and re-plating steps used in the porcine system are generally excluded from the human and mouse systems. Due to the aforementioned disadvantages of the current porcine MoDC generation protocol, our study aimed to improve a porcine MoDC generation protocol by modifying the current conventional protocol, referred to as the modified protocol, based on human MoDC and murine BM-DC culture procedures. This work will be beneficial for the study of immunobiology of porcine DCs and mechanisms of host-pathogen interactions. The validated MoDCs will also be expedient for future research works in antigen delivery, vaccines and immunotherapy in pigs, as well as relevant species.

2. Material and methods

2.1. Animal

Eight-week-old, crossbred pigs were subjected to whole blood collection. The heparinized blood samples were kept in at 4 °C during transportation, and processed within 4 h following blood collection. All animal procedures were reviewed and approved by Chulalongkorn University Animal Care and Use Committee (protocol number 1631029).

2.2. In vitro *generation and stimulation of porcine MoDC*

Porcine PBMCs were isolated from heparinized whole blood by density gradient centrifugation using Lymphosep® separation medium (Biowest, Kansas, Mo, USA) according to previously described protocol (Suradhat and Thanawongnuwech, 2003).

For the conventional protocol, 25×10^6 porcine PBMCs were plated in 1 ml of IMDM containing 5% fetal bovine serum (FBS) in a well of 6-well-plate for 2 h, followed by removal of the non-adherent cells. The adhered cells, referred as monocytes, were subsequently cultured with 1 ml of RPMI containing 10% FBS, 2 mM L-glutamine, antibiotic/antimycotic solution, 25 mM HEPES (all above reagents were obtained from GIBCO, Carlsbad, CA, USA) and 50 μM β-mercaptoethanol (Sigma Chemical Co., St. Louis, MO, USA), and supplemented with 10 ng/ml and 25 ng/ml recombinant porcine IL-4 and GM-CSF (R&D system, Minneapolis, MN, USA) respectively, referred as DCs medium, for 7 days. Every 2 days, the culture medium was 50% replaced with fresh DCs medium. At day 7, MoDCs were harvested and re-plated for 1×10^6 per ml of DCs medium in a well of 24-well-plate prior to DCs activation experiment.

For the modified protocol, 5×10^6 PBMCs in 1 ml of IMDM containing 5% FBS were plated in a well of 24-well-plate. Non-adherent cells were removed, and the adhered cells were further cultured with DCs medium for 7 days. Every 2 days, the culture medium was 50% replaced with fresh DCs medium. For downstream *in vitro* studies, the MoDCs were incubated with stimulus without harvesting and re-plating step.

The cells from both protocols were cultured at 37 °C in a 5% CO_2 incubator. For LPS stimulation, MoDCs were stimulated with 0.1 mg/ml LPS (Sigma aldrich) for 24 h. Un-stimulated MoDCs were used as the negative control.

2.3. Percentage of adhered monocyte and number of adhered MoDCs

Following PBMC plating and removal of non-adherent cells, the remaining adhered cells were harvested and enumerated using Z2 Coulter counter (Beckman Coulter, CA, USA). Percentage of the cells was calculated by (number of the adhered cells/total number of PBMCs) × 100. In addition, the 7-day cultured MoDCs were harvested and examined the cell number using the same protocol.

2.4. MoDC morphology

The morphology of cultured monocytes and MoDCs at day 7 were observed under the inverted bright field microscopy (Olympus), at 40× stage objectives.

2.5. Phagocytosis assay

The phagocytosis assay was performed as previously described (Dewitte et al., 2014). MoDCs (5×10^5 cells/well) were incubated with FITC-conjugated *Escherichia coli* (Molecular Probes, Invitrogen, USA) at 1:50 ratio of MoDC: *E. coli*, at 37 °C for 10 min with shaking. Then, cold PBS was added to stop phagocytic activity, and the cells were washed 4 times. The MoDCs were then resuspended in FACs buffer for flow cytometric analysis.

2.6. Flow cytometric analyses

For surface markers staining, Primary antibodies included anti-porcine CD1-PE (76-7-4), anti-porcine SLA-DR (1053H2-18), biotinylated-anti-porcine CD172a (74-22-15) were obtained from SouthernBiotech (Birmingham, AL, USA), and anti-human CD86-PEcy7 (IT2.2) and the isotype control antibody (MPC-11) were obtained from BioLegend (San Diego, CA, USA). Secondary antibodies including goat anti-mouse IgG2a, κ-FITC and streptavidin-APC were obtained from Invitrogen (Carlsbad, CA, USA). For intracellular staining, MoDCs were stained with biotinylated-anti-porcine IFN-γ (P2C11, IgG2a) (BD Biosciences, San Diego, CA, USA) and streptavidin-PE/cy7, or IgG2a and isotype control (BioLegend). Subsequently, the stained cells were analyzed by Beckman FC550 (Beckman Coulter), and FlowJo software.

2.7. Quantitative real-time polymerase chained reaction (qRT-PCR)

Following LPS stimulation, total mRNA was extracted from the MoDCs using UPzol™ (Biotechrabbit, Germany), and converted to cDNA by Supercript III First-Strand cDNA synthesis kit (Invitrogen). The qRT-PCR reaction was performed as previous described (Wongyanin et al., 2012). The specific primers used were *IL1B* (IL-1β), F5′- AAC-GTG-CAA-TGA-TGA-CTT-TG-3′ and R5′- CAC-TTC-TCT-CTT-CAA-GTC-CC-3′; *IL6* (IL-6), F5′- AGA-ACT-CAT-TAA-GTA-CAT-CCT-CG and R5′- AGA-TTG-GAA-GCA-TCC-GTC-3′; *IL12A* (IL-12p35), F5′- ATG-CCT-CAA-CCA-CTC-CCA-AA-3′ and R5′- GGC-AAC-TCT-CAT-TCG-TGG-CT-3′; *IL12B* (IL-12p40), F5′- CCC-TGG-AGA-AAT-GGT-GGT-CC-3′ and R5′- GGC-CAG-CAT-CTC-CAA-ACT-CT-3′; *IFNA* (IFN-α), F5′- CAC-CAC-AGC-TCT-TTC-CAT-3′ and R5′- CAC-CAC-AGC-TCT-TTC-CAT-3′; *IFNB* (IFN-β), F5′- CTG-GAG-GAG-GAC-TCC-AT-3′ and R5′- GAG-TCT-GTC-TTG-CAG-GTT-3′; *IFNG* (IFN-γ), F5′- GAG-GTT-CCT-AAA-TGG-TAG-CTC-3′ and R5′-GTC-TGA-CTT-CTC-TTC-CGC-3′; *GAPDH* (GAPDH), F5′- AAG-TGG-ACA-TTG-TCG-CCA-TC-3′ and R5′- TCA-CAA-ACA-TGG-GGG-CAT-C-3′. The Ct values of the target gene were normalized against those of the housekeeping gene; *GAPDH*. Differences in Ct values between the treatments were analyzed by the formula $2^{-(\Delta CP\text{target gene} - \Delta CP\text{gapdh})}$, using a Rotor-Gene Real-Time Analysis Software 6.0 (Corbett Research).

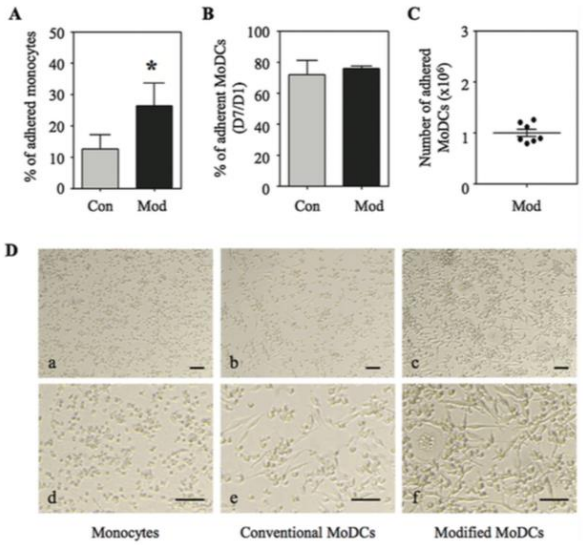

Fig. 1. Percentage of adhered monocytes and morphology of monocytes and MoDCs.
(A) The percentage of adhered monocytes prior to MoDC induction in the conventional and modified protocol was determined by calculating the ratio of adhered monocytes per total PBMCs. (B) MoDCs were generated from two protocol and the proportion of adherent MoDCs on day 7 over day 1 was calculate as the percentage. (C) Total number of 7-day cultured MoDCs generated by the modified protocol from seven individual samples. $^{*}p < 0.05$ comparing between MoDCs from both protocols. Con, conventional protocol; Mod, modified protocol. (D-a, d) Porcine peripheral blood monocytes were cultured in non-DC medium and MoDCs generated from the (D-b, e) conventional and (D-c, f) modified protocols were cultured in DC medium. At day 7, cell morphology was observed under bright field microscopy (40x). Scale bars, 50 μm.

Table 1
Comparison of conventional and modified protocol.

Culture procedure	Conventional	Modified
Number of initial PBMCs per well	25×10^6 cells	5×10^6 cells
Culture ware	6-well plate	24-well plate
Volume of culture medium	1 ml	1 ml
Cytokines	25 ng/ml GM-CSF 10 ng/ml IL-4	25 ng/ml GM-CSF 10 ng/ml IL-4
Harvesting step	Yes	No
Re-plating step	Yes	No

3. Results and discussion

3.1. The modified MoDC generation protocol increased the number of adherent monocytes, but still maintained dendritic cell-like morphology

The generation of MoDCs by the conventional and modified culture procedures differed in two steps: the initial plating and re-plating (Table 1). At the initial step, the PBMC number in the modified protocol was 5-fold less than the conventional protocol; however, the percentage of adherent monocytes in the modified protocol was obviously greater than those in the conventional protocol (Fig. 1A). During the culture period, the cells from both procedures retained approximately 70–80% attachment (Fig. 1B). Compared with the original protocol, the modified protocol required fewer PBMCs to generate the same amount of MoDCs (Fig. 1A–B). It is possible that the lower number of PBMCs in the cultured system improved the ratio of cells to contact surface area on the culture plate leading to enhanced monocyte adherence.

The purpose of re-plating MoDCs in the conventional protocol was to control the accuracy of cell numbers, however, these steps may induce premature DC differentiation as a consequence of mechanical stimulation (Craig et al., 2009) leading to reduced phagocytosis ability and cytokine production in response to stimuli (Mellman and Steinman, 2001), which could affect the accuracy and reliability of the downstream experiment. Thus, the re-plating step was excluded from our modified protocol. After the 7-day-culture, the number of MoDCs generated from the modified protocol was consistent among the individual wells (Fig. 1C), indicating that the re-plating step is dispensable for MoDC culture.

The morphology of porcine MoDCs has been well characterized by cytoplasmic projections around the cell surface (Lecours et al., 2011; Lin et al., 2012). We therefore observed the change in cell morphology at the end of the culture period. All MoDCs elongated and transformed to DC-like morphology (Fig. 1Db–c and De–f), which was absent in the monocytes (Fig. 1Da, d). In addition, MoDCs from the modified protocol (Fig. 1Dc, f) were phenotypically similar to those from the conventional protocol (Fig. 1Db, e), both in size and morphology.

3.2. MoDCs generated from the modified protocol highly expressed the dendritic cell marker, CD1 molecule, and had increased phagocytic activity

To characterize phenotypes of the obtained MoDCs, surface expressions of CD1, SLA-DR and CD172a markers were determined (Fig. 2A–B). The CD1 molecule has been recognized as a lipid presen-

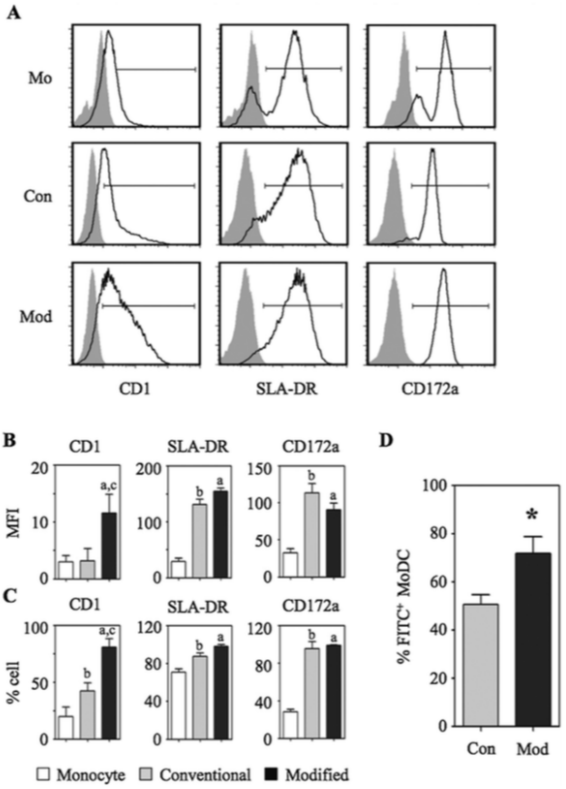

Fig. 2. Comparative analysis of MoDC phenotype and phagocytic activity.
Monocytes and immature MoDCs generated from the modified and conventional protocols were harvested at day 7. (A–C) The cells were stained against CD1, CD172a and SLA-DR and analyzed by flow cytometry. (A) Histogram demonstrating the expression level of DC marker (thick line) and isotype control staining (gray shade). Data are representative of three independent experiments. (B) Mean fluorescent intensity (MFI) and (C) the percentage of positive cells of CD1, CD172a and SLA-DR in monocytes and MoDCs from the conventional and modified protocols were determined. (D) MoDCs obtained from each protocol were cultured with FITC-labeled *E. coli*. The percentage of FITC positive cells, representing MoDCs engulfing *E. coli*, were analyzed by flow cytometry. $^{a}p < 0.05$ when comparing MoDCs from the modified protocol to monocytes; $^{b}p < 0.05$ when comparing MoDCs from the conventional protocol to monocytes; $^{c}p < 0.05$ when comparing MoDCs from the modified protocol to those of the conventional protocol. $^{*}p < 0.05$ when comparing MoDCs from the modified protocol to those of the conventional protocol; n = 5 pigs. Mo, monocytes; Con, conventional protocol; Mod, modified protocol. Two different MoDC generation protocols were performed concurrently using PBMCs from the same animals. Data are representative of two independent experiments.

tation molecule solely expressed in the DC population. In addition, CD1 is not constitutively expressed by monocytes, but is upregulated during monocyte differentiation into DCs (Summerfield and McCullough, 2009). Notably, MoDCs from the modified protocol highly expressed CD1 molecules, while the MoDCs from the conventional protocol expressed CD1 at a comparable level to the monocytes (Fig. 2A–C, left panel). Furthermore, the $CD1^{+}$ population was markedly present in MoDCs from the modified protocol, but was only moderately present in MoDCs obtained from the conventional protocol (Fig. 2A–C, left panel).

The obtained MoDCs from both protocols showed upregulated expression of SLA-DR and CD172a and an elevated percentage of $SLA\text{-}DR^{+}$ and $CD172a^{+}$ populations when compared to the monocytes (Fig. 2B–C, middle and right panels). However, there was no statistical difference in the SLA-DR and CD172a expression level and $SLA\text{-}DR^{+}$ and $CD172a^{+}$ cells between the two protocols (Fig. 2B–C, middle and right panel).

The elevation of CD1 expression and $CD1^{+}$ cells in MoDCs obtained from the modified protocol possibly indicates an increase in DC differentiation. To confirm whether these DCs were able to function, we assessed phagocytosis activity by using FITC-labeled *Escherichia coli* as an antigen. Comparing between the two culture protocols, MoDCs derived from the modified protocol displayed a higher efficacy of *E. coli* engulfment (Fig. 2D).

The MoDCs obtained from the modified protocol expressed the DC markers, CD1, CD172a and SLA-DR, similar to those generated by the conventional protocol, concordant with previous reports (Chamorro et al., 2005; McCullough et al., 1997; Summerfield and McCullough, 2009). Nevertheless, the MoDCs obtained from the modified protocol showed an increase in $CD1^{+}$ cells (Fig. 2A–C) and

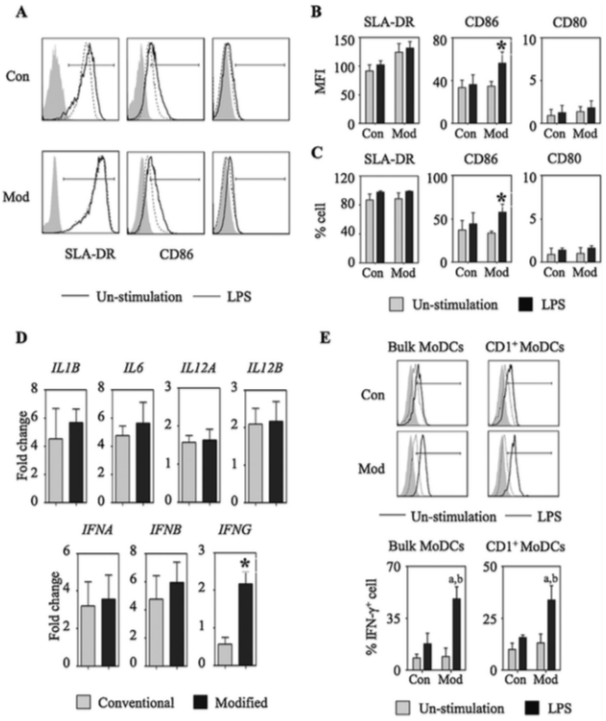

Fig. 3. The expressions of DC maturation markers and pro-inflammatory cytokines.
MoDCs generated from the conventional and modified protocols were stimulated with 1 μg/ml LPS for 24 h. (A–C) The cells were stained for SLA-DR, CD80 and CD86 and subjected to flow cytometric analyses. (A) Histograms demonstrating the expression level of the activation marker from LPS stimulation (thick line) and non-stimulated (dashed line) MoDCs. Isotype control was used for the negative staining (grey shade). Data are representative of three independent experiments. The (B) mean fluorescent intensity (MFI) and (C) percentage of positive cells of SLA-DR, CD80 and CD86 in monocytes and MoDCs from the conventional and modified protocols were determined. (D) The expressions of the *IL1B*, *IL6*, *IL12A*, *IL-12B*, *IFNA*, *IFNB* and *IFNG* genes were evaluated by qRT-PCR. The relative gene expression was shown as the fold induction over the non-stimulated MoDCs. $^{*}p < 0.05$ when comparing LPS stimulated MoDCs from the modified protocol to those of the conventional protocol. (E) Histogram demonstrating the level of IFN-γ expression and isotype control staining (gray shade). The percentage of IFN-γ producing cells was determined by flow cytometric analysis from bulk MoDCs and CD1^{+} MoDC populations. $^{a}p < 0.05$ when comparing LPS stimulated MoDCs to non-stimulated MoDCs from the conventional protocol and $^{b}p < 0.05$ when comparing LPS stimulated MoDCs from the modified protocol to those of the conventional protocol; n = 3 pigs. Con, conventional protocol; Mod, modified protocol. Two different MoDC generation protocols were performed concurrently using PBMCs from the same animals. Data are representative of two independent experiments.

enhanced phagocytic activities (Fig. 2D) when compared to those from the conventional protocol, indicating a more efficient generation of porcine MoDCs by the modified protocol. As the downstream signals and the optimal doses of GM-CSF and IL-4 are mandatory for MoDC differentiation *in vitro* (Heystek et al., 2000; Menges et al., 2005), it is possible that the lower number of cultured monocytes per well in the modified protocol allowed better utilization of the supplements, leading to the higher quantity and quality of the generated porcine MoDCs. In addition, the omitted mechanical stimulation in the modified protocol could have contributed to the more efficient DC generation.

3.3. MoDCs generated from modified protocol were capable of undergoing maturation

According to the efficient DC differentiation obtained from the modified protocol, we next questioned whether these MoDCs were able to undergo maturation. To evaluate the DC maturation stage, the cultured MoDCs were stimulated with LPS, and the expression of maturation markers SLA-DR, CD80 and CD86 were determined (Fig. 3). From flow cytometric analysis, all MoDCs altered their phenotype upon LPS stimulation by upregulating the expression of SLA-DR and CD86, but not CD80, molecules (Fig. 3A–C). The frequency and level of maturation markers in MoDCs from the modified protocol were similar to those from the conventional protocol (Fig. 3B and C). The data suggests that our modified culture procedure was capable of generating potent MoDCs *in vitro*.

3.4. The modified protocol improved effector properties of mature MoDCs

Generation of porcine MoDCs is not only beneficial for the study of porcine DC biology, but is also essential for *in vitro* tests in clinical application. *In vitro* generated DCs, therefore, must

represent *in vivo* DCs in both cellular phenotype and effector properties. To investigate the effector properties of mature MoDCs, the MoDCs were stimulated with LPS and the expression of pro-inflammatory cytokine genes (Fig. 3D) was determined. Upon LPS stimulation, *IL1B*, *IL6* and *IFNB* mRNA, encoding IL-1β, IL-6 and IFN-β, respectively, were induced in MoDCs generated from the modified protocol at levels comparable to those from the conventional protocol. Interestingly, the expression level of the *IFNG* gene, encoding IFN-γ, was significantly upregulated in MoDCs obtained from the modified protocol (Fig. 3D). To confirm the production of IFN-γ protein levels, LPS-stimulated MoDCs were subjected to flow cytometric analyses (Fig. 3E). Consistent with results from the gene expression study, upon LPS stimulation, the level of IFN-γ production was greatly increased in both bulk and $CD1^+$ in MoDCs obtained from the modified protocol, while IFN-γ production in the MoDCs obtained from the conventional protocol was only slightly increased.

Of interest, MoDCs generated by the modified protocol displayed high levels of *IFNG* gene expression and IFN-γ production upon maturation (Fig. 3D and E). Interferon-gamma is not only important for Th1 polarization but is also essential for driving DC maturation and endowing the effector properties of DCs (Frasca et al., 2008; Han et al., 2009; He et al., 2007; Pan et al., 2004). Dendritic cells produce high levels of IFN-γ in response to stimuli, especially LPS (Fedele et al., 2008; Pan et al., 2004). Interferon-gamma receptor deficient BM-DCs exhibited decreased expression of the maturation marker, CD86, reduced production of the pro-inflammatory cytokines, IL-1β and IL-12, and impaired function to activate alloreactive T cells (Pan et al., 2004). In addition, exogenous IFN-γ enhanced DC maturation and function (Frasca et al., 2008; Han et al., 2009; Moretto et al., 2007). Interferon-gamma producing DCs potentially induced the antigen-specific cytotoxic T cell responses both *in vitro* and *in vivo* (He et al., 2007; Lemoine et al., 2010; Moretto et al., 2007). Interferon-gamma thus acts as an autocrine mediator for DC maturation and exerts T cell effector functions. Our findings suggest that the modified protocol improved MoDC maturation and effector properties. This advantage will be useful for future *in vitro* tests and clinical applications.

4. Conclusion

The development of MoDCs in the culture system provides great advantages to studies involving DC immunobiology, especially regarding the host-pathogen interaction. Here, we proposed an *in vitro* MoDC generation protocol that required fewer porcine PBMCs and yielded a good quantity and quality of porcine MoDCs. The modified protocol has potential for numerous future applications and will be a valuable platform for future research in antigen delivery, vaccines and immunotherapy in pigs, as well as relevant veterinary species.

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Appendix B

Interleukin-1 receptor antagonist: an early immunomodulatory cytokine induced by porcine reproductive and respiratory syndrome virus

Teerawut Nedumpun,[1] Piya Wongyanin,[2] Chaitawat Sirisereewan,[3] Patcharee Ritprajak,[4] Tanapat Palaga,[5] Roongroje Thanawongnuwech[6,7] and Sanipa Suradhat[7,8,*]

[1] Interdisciplinary Program of Medical Microbiology, Graduate School, Chulalongkorn University, Bangkok 10330, Thailand

[2] Department of Medical Technology, Faculty of Science and Technology, Bansomdejchaopraya Rajabhat University, Bangkok 10330, Thailand

[3] Graduate Program in Veterinary Pathobiology, Faculty of Veterinary Science, Chulalongkorn University, Bangkok 10330, Thailand;

[4] Department of Microbiology, RU in Oral Microbiology and Immunology, Faculty of Dentistry, Chulalongkorn University, Bangkok 10330, Thailand

[5] Department of Microbiology, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand

[6] Department of Veterinary Pathology, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, 10330 Thailand

[7] Center of Excellence in Emerging Infectious Diseases in Animals, Chulalongkorn University (CU-EIDAs), Bangkok 10330, Thailand

[8] Department of Veterinary Microbiology, Faculty of Veterinary Science, Chulalongkorn University, Bangkok 10330, Thailand.

* Corresponding author:

Professor Sanipa Suradhat

Mailing address: Department of Veterinary Microbiology, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, 10330, Thailand.

E-mail address: Sanipa.S@chula.ac.th (S. Suradhat)

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Interleukin-1 receptor antagonist: an early immunomodulatory cytokine induced by porcine reproductive and respiratory syndrome virus

Teerawut Nedumpun,[1] Piya Wongyanin,[2] Chaitawat Sirisereewan,[3] Patcharee Ritprajak,[4] Tanapat Palaga,[5] Roongroje Thanawongnuwech[6,7] and Sanipa Suradhat[7,8,*]

Abstract

Porcine reproductive and respiratory syndrome virus (PRRSV) infection poorly induces pro-inflammatory cytokines (IL-1, IL-6 and TNF-α) and type I IFN production during the early phase of infection. Our microarray analysis indicated strong upregulation of the *IL1RA* gene in type 2 PRRSV -infected monocyte-derived dendritic cells. Interleukin-1 receptor antagonist (IL-1Ra) is an early inhibitory cytokine that suppresses pro-inflammatory cytokines and T-lymphocyte responses. To investigate the induction of IL-1Ra by PRRSV, monocyte-derived dendritic cells were cultured with type 2 PRRSV or other swine viruses. PRRSV increased both *IL1RA* gene expression and IL-1Ra protein production in the culture. The enhanced production of IL-1Ra was further confirmed in PRRSV-cultured PBMC and PRRSV-exposed pigs by flow cytometry. Myeloid cell population appeared to be the major IL-1Ra producer both *in vitro* and *in vivo*. In contrast to the type 2 PRRSV, the highly pathogenic (HP)- PRRSV did not upregulate *IL1RA* gene expression *in vitro*. To determine the kinetics of PRRSV-induced *IL1RA* gene expression in relation to other pro-inflammatory cytokine genes, PRRSV-negative pigs were vaccinated with a commercially available type 2 modified-live PRRS vaccine or intranasally inoculated with HP-PRRSV. In modified-live PRRS vaccine pigs, upregulation of *IL1RA*, but not *IL1B* and *IFNA*, gene expression was observed from 2 days post- vaccination. Consistent with the *in vitro* findings, upregulation of *IL1RA* gene expression was not observed in the HP-PRRSV-infected pigs throughout the experiment. This study identified IL-1Ra as an early immunomodulatory mediator that could be involved in the immunopathogenesis of PRRSV infections.

INTRODUCTION

Innate immunity is critical for induction of virus-specific immune responses [1]. Pro-inflammatory cytokines (IL-1, IL-6 and TNF-α) and antiviral cytokines [type I interferon (IFN)] are innate cytokines, which help promote leukocyte recruitment and inhibition of viral replication [2–6]. Porcine reproductive and respiratory syndrome virus (PRRSV) is one of the major swine pathogens that causes serious economic losses in the swine production industry worldwide [7]. PRRSV can be classified into two genotypes: type 1 (EU) and type 2 (US) strains [8]. PRRSV infection usually induces poor innate and adaptive immune responses [9, 10]. In addition, PRRSV infection usually enhances secondary infections in the lungs, leading to porcine reproductive and respiratory disease complex [11] or vaccination failure [12–15]. The findings indicated that PRRSV infection could affect overall immunocompetency of the infected pigs. Recently, the emergence of the variant PRRSV strain, known as a highly pathogenic (HP)-PRRSV, has been reported [16]. The clinical manifestation of HP-PRRSV is different from that of the typical PRRSV strains, causing severe clinical outcomes and high mortality rate among the

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Author affiliations: [1]Interdisciplinary Program of Medical Microbiology, Graduate School, Chulalongkorn University, Bangkok, Thailand; [2]Department of Medical Technology, Faculty of Science and Technology, Bansomdejchaopraya Rajabhat University, Bangkok, Thailand; [3]Graduate Program in Veterinary Pathobiology, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand; [4]Department of Microbiology, RU in Oral Microbiology and Immunology, Faculty of Dentistry, Chulalongkorn University, Bangkok, Thailand; [5]Department of Microbiology, Faculty of Science, Chulalongkorn University, Bangkok, Thailand; [6]Department of Veterinary Pathology, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand; [7]Center of Excellence in Emerging Infectious Diseases in Animals, Chulalongkorn University (CU-EIDAs), Bangkok, Thailand; [8] Department of Veterinary Microbiology, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand.
***Correspondence:** Sanipa Suradhat, sanipa.s@chula.ac.th
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Abbreviations: CSFV, classical swine fever virus; CU-VDL, Chulalongkorn University Veterinary Diagnostic Laboratory; HP, highly pathogenic; IFN, interferon; IL-1Ra, interleukin-1 receptor antagonist; LPS, lipopolysaccharide; MLV, modified-live PRRS vaccine; MoDC, monocyte-derived dendritic cell; PBL, peripheral blood lymphocyte; p.i., post-infection; PRRSV, porcine reproductive and respiratory syndrome virus; p.v., post-vaccination; qPCR, quantitative real-time PCR; sIg, surface immunoglobulin; Treg, regulatory T cell.

infected pigs [17, 18]. In contrast to the typical PRRSV, infection of HP-PRRSV resulted in strong pro-inflammatory cytokine production in the infected host [19, 20]. However, the underlying mechanisms of the differences in the pathogenicity of the two viruses are not fully understood.

PRRSV primarily infects monocytes/macrophages and dendritic cells, resulting in inhibited expression of MHC and co-stimulatory molecules in the infected cells [21–23]. Moreover, PRRSV infection promotes apoptosis of the infected cells [24, 25]. In the early phase of infection, PRRSV induces delayed and weak pro-inflammatory cytokines, including IL-1, IL-6 and TNF-α and type I IFN production [26–29]. It has also been reported that PRRSV suppressed the production of IL-1β, IL-8 and IFN-α in serum during the first 14 days post-infection (p.i.) [30]. In the infected lungs, decreased IL-1, IL-6 and TNF-α production was also observed during the first 10 days of PRRSV infection [28]. Some studies suggested that the non-structural proteins and nucleocapsid protein of PRRSV could modulate type I IFN production *in vitro* [31–33]. These findings clearly indicated that PRRSV infection significantly interferes with the innate immune functions. In addition, recent publications indicated that type 2 PRRSV infection could induce IL-10 production and viral-specific regulatory T cells (Treg) both *in vitro* and *in vivo* [10, 34–37], which could lead to the inefficient induction of viral-specific immune responses. Interestingly, some of the type 1 PRRSV strains did not induce IL-10 and Treg production, yet still exhibited clinical and immunological manifestation as do type 2 PRRSV [38, 39]. Thus, the immunosuppressive mechanism of PRRSV during the early phase of PRRSV infection remains incompletely understood.

Interestingly, our preliminary microarray analysis demonstrated that type 2 PRRSV-infected monocyte-derived dendritic cells (MoDCs) exhibited strong upregulation of interleukin-1 receptor antagonist (IL-1Ra) gene expression (unpublished observation). IL-1Ra is an early anti-inflammatory cytokine that controls inflammatory responses during an early stage of immune activation [40]. IL-1Ra competitively binds to the interleukin-1 receptor and subsequently blocks the intracellular IL-1 signalling cascade [41]. IL-1Ra is produced by monocytes, macrophages or dendritic cells, which are known to be PRRSV target cells [22, 41, 42]. IL-1Ra can modulate the production of IL-1 and TNF-α [43] and type I IFN [44]. Therefore, early IL-1Ra production could affect induction of pro-inflammatory and antiviral cytokines during the early phase of PRRSV infection. In this study, we aimed to confirm and investigate the induction of IL-1Ra by type 2 PRRSV strains, including the variant HP-PRRSV, using both *in vitro* and *in vivo* infection models.

RESULTS

PRRSV upregulated *IL1RA* gene expression in cultured porcine MoDCs and PBMCs

To investigate the factors involved in virus-induced immunomodulation during the early phase of PRRSV infection, porcine MoDCs were cultured in the presence of type 2 PRRSV (01NP1) for 24 h, and the total RNA was subjected to microarray analysis using the Porcine_GXP_4×44K (two-colour microarray-based gene expression platform). The relative expression of the innate, T-helper polarizing and inhibitory cytokine genes is shown in Fig. 1(a) (upper panels). Strong upregulation of genes *IL10* and *IL1RA* was clearly evident. Upregulations of genes *IFNA*, *IFNB*, *IL1B* and *IL2* were also observed. The *in vitro* findings on upregulation of type I IFN gene were consistent with previous reports [45, 46]. Interestingly, while most of the type I IFN receptor downstream signalling genes were upregulated, consistent with the enhanced type I IFN gene expression, upregulation of the IL-1 receptor downstream signalling genes was not evident in the system (Fig. 1a, lower panels). The results indicated that the presence of PRRSV upregulated expression of *IL1RA* and *IL1B*, but not the IL-1 receptor downstream signalling genes, in the cultured MODC.

To confirm the above findings, the levels of *IL1RA* gene expression in the MoDC cultured in the presence of type 2 PRRSV (01NP1) or other swine respiratory viruses were analysed using quantitative real-time PCR (qPCR). Classical swine fever virus (CSFV) and swine influenza virus, which had been reported to induce strong pro-inflammatory cytokines (IL-1, IL-6 and TNF-α) and type I IFN production during the early phase of infection [47–49], were also included in the experiment. The levels of *IL1RA* gene expression determined by qPCR are shown in Fig. 1(b). The results demonstrated that the presence of PRRSV, but not other swine viruses, significantly enhanced *IL1RA* gene expression in the cultured MoDC. In addition, it should be pointed out that PRRSV-induced *IL1RA* gene expression was much stronger than that from the cells treated with lipopolysaccharide (LPS) (Fig. 1b). The kinetics of PRRSV-induced *IL1RA* gene expression in the cultured porcine PBMC were investigated and are shown in Fig. 1(c). The percentages of PRRSV cells in the myeloid subpopulation (SWC3 cells) after 3, 6, 12 and 24 h incubation were 12.2 ±1.6 %, 14.4±3.2 %, 24±2.6 % and 30.1±2.9 %, respectively. Upregulation of the *IL1RA* gene was evident from 3 h and peaked at 12 h following infection with type 2 PRRSV (Fig. 1c). Interestingly, the level of *IL1RA* gene expression in the PRRSV-infected PBMC remained higher than that from LPS treatment at 24 h (Fig. 1c). The data suggested that the presence of PRRSV enhanced and prolonged *IL1RA* gene expression in the culture system.

To confirm the gene expression experiments, the levels of IL-1Ra protein in the cultured supernatants were determined by ELISA. Consistent with the aforementioned results, the presence of PRRSV enhanced IL-1Ra production in the cultured MoDC (Fig. 2a). The levels of IL-1Ra protein detected in the supernatants correlated well with those of *IL1RA* gene expression (r^2=0.7664, P<0.001) (Fig. 2b).

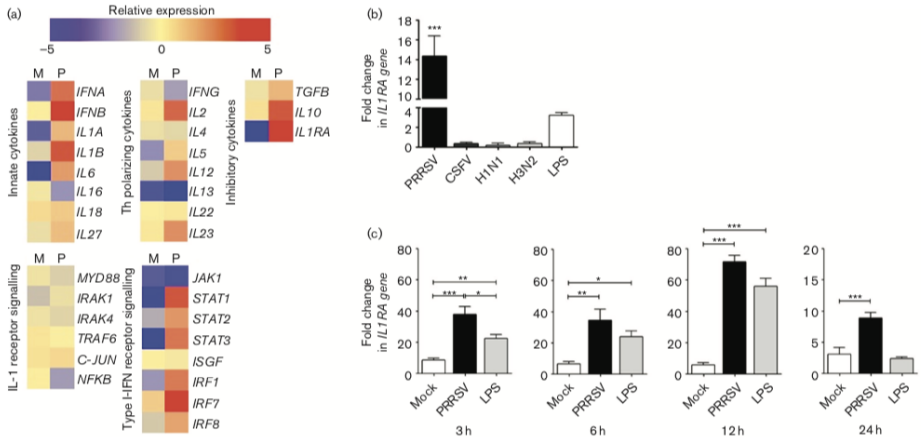

Fig. 1. PRRSV upregulated *IL1RA* gene expression in the cultured porcine leukocytes. (a) Microarray analysis of cytokine genes and related cytokine signalling genes between the MODC treated with type 2 PRRSV (P) and mock-infected MoDC (M) was compared. Porcine MoDCs were cultured in the presence of 0.1 m.o.i. of type 2 PRRSV (01NP1) or MARC-145 cell lysate for 24 h. (b) PRRSV significantly upregulated *IL1RA* gene expression. Porcine MoDCs (five pigs per group) were cultured with type 2 PRRSV (01NP1), classical swine fever virus (CSFV), H1N1, H3N2, lipopolysaccharide (LPS) or the relevant mock-infected cell lysate for 24 h. Data represent the fold changes in the levels of *IL1RA* gene expression obtained from the treatment subtracted by those of the relevant mock-infected cell lysate. (c) Kinetics of *IL1RA* gene expression in the PRRSV- and mock-infected PBMC were performed at 3, 6, 12 and 24 h. PBMCs were cultured with 0.1 m.o.i. of type 2 PRRSV (01NP1) or MARC-145 cell lysate or 0.1 μg ml^{-1} LPS (five pigs per group), then harvested at the indicated time point. ***, ** and * indicate significant difference at $P<0.0001$, $P<0.001$ and $P<0.05$, respectively.

The myeloid population was responsible for IL-1Ra production in the PRRSV infection model

To determine the porcine leukocyte subpopulation responsible for IL-1Ra production in this model, various porcine leukocyte subpopulations were cultured *in vitro* with type 2 PRRSV for 24 h. The presence of type 2 PRRSV resulted in significant upregulation of *IL1RA* gene expression in the cultured MoDC and PBMC, but not in the peripheral blood lymphocyte (PBL) subpopulation (Fig. 3a). The IL-1Ra protein production by porcine leukocytes was further confirmed by flow cytometry. The cultured PBMCs were initially gated into myeloid and lymphocyte subpopulations based on the forward and side scatter profiles. The cells were further identified by the expression of SWC3 (myeloid), CD3 (T-lymphocyte) and surface immunoglobulin (sIg) (B-lymphocyte) markers. The identified lymphocyte subpopulations consisted of the cells that highly expressed CD3 (T lymphocytes) and sIg (B lymphocytes), while the myeloid subpopulation primarily expressed the SWC3, but not CD3 and sIg, marker (Fig. 3b). The numbers of myeloid cells obtained from the cells treated with PRRSV and mock-infected PBMC were insignificant (data not shown). The results indicated that the presence of type 2 PRRSV significantly enhanced IL-1Ra production in the myeloid, but not lymphocyte subpopulations (Fig. 3c, d). Together, the results indicated that porcine myeloid population is the major IL-1Ra producer in this culture system, and the presence of PRRSV significantly enhanced IL-1Ra production from this cellular population.

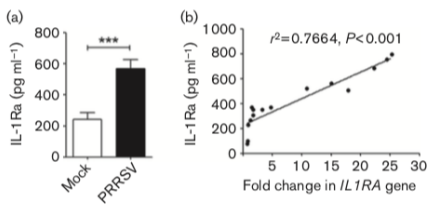

Fig. 2. PRRSV-induced IL-1Ra production correlated well with the levels of *IL1RA* gene expression. (a) Levels of secretory IL-1Ra in the MoDC cultured supernatants. (b) Correlation of the levels of IL-1Ra detected by ELISA and those of *IL1RA* gene expression. Porcine MoDC (eight pigs per group) were cultured with 0.1 m.o.i. of type 2 PRRSV (01NP1) or MARC-145 cell lysate for 24 h. *** indicates significant difference at $P<0.0001$.

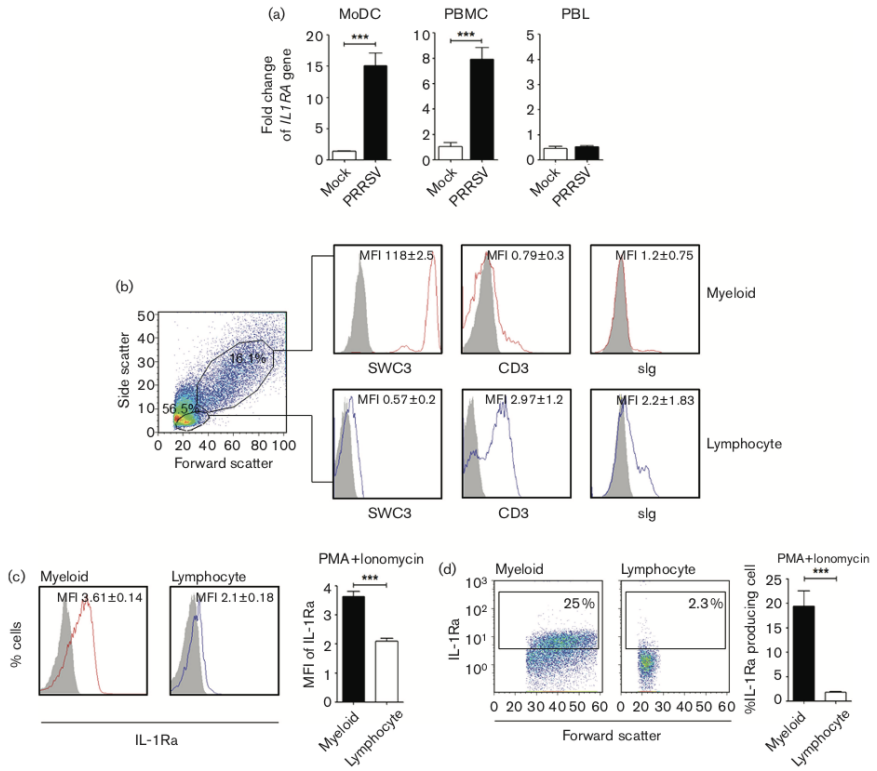

Fig. 3. PRRSV enhanced IL-1Ra production in the myeloid subpopulation *in vitro*. (a) PRRSV upregulated *IL1RA* gene expression in the cultured MoDC and PBMC, but not PBL. (b) Characteristics of porcine myeloid and lymphocyte subpopulations. Porcine leukocyte subpopulations were identified based on the expression of the surface markers; SWC3 (myeloid cells), CD3 (T lymphocytes) and sIg (B lymphocytes). (c) Mean fluorescent intensity (MFI) of IL-1Ra production and (d) Percentages of IL-1Ra-producing cells in the indicated leukocyte subpopulations. Porcine PBMC (five pigs/group) were cultured with 0.1 m.o.i. of type 2 PRRSV (01 NP1) or MARC-145 cell lysate for 48 h. Data represent the MFI or percentage of IL-1Ra-producing cells obtained from sample treated with PRRSV subtracted by that of mock-infected PBMC. *** indicates significantly different at $P<0.0001$.

PRRSV infection increased numbers of IL-1Ra producing cells in infected pigs

To confirm the induction of IL-1Ra by PRRSV *in vivo*, PRRSV-seronegative pigs were vaccinated with the commercially available type 2 modified-live PRRS vaccine (MLV), which has previously been shown to possess immunomodulatory properties similar to the field strain PRRSV [50, 51]. On days 0, 4, 7 and 14 post-vaccination (p.v.), PBMCs were collected from the immunized pigs to assess the numbers of IL-1Ra-producing cells by flow cytometry. The myeloid and lymphocyte subpopulations were gated and identified, based on the expression of SWC3, CD3 and sIg surface markers (see above) (Fig. 4a). The percentages of the myeloid subpopulation obtained from the MLV and PBS control groups were comparable (data not shown). Consistent with the *in vitro* findings, immunization with type 2 PRRS-MLV significantly enhanced IL-1Ra production in the myeloid subpopulation (Fig. 4b, c). Viral genomic RNA was detected from 4 days p.v. and remained high throughout the observation period, confirming ongoing PRRSV infection in the vaccinated pigs (Fig. 4d). The kinetics of IL-1Ra production between the experimental groups

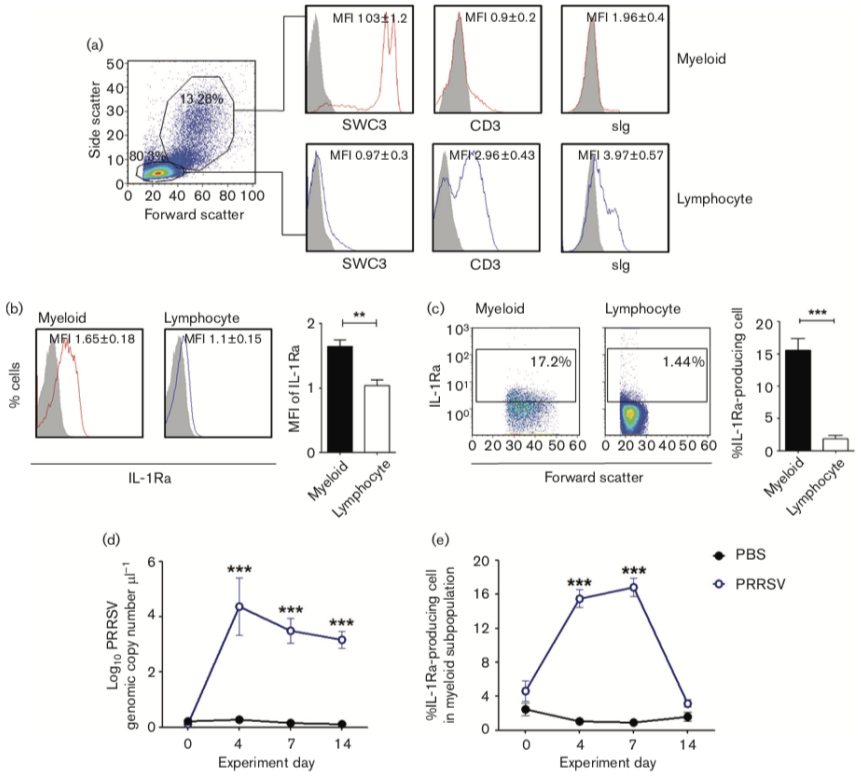

Fig. 4. PRRSV enhanced IL-1Ra-producing cells in pigs receiving PRRS-MLV. (a) Characteristics of the myeloid and lymphocyte subpopulations in the PBMC of the pigs receiving PRRS-MLV. (b) Mean fluorescent intensity (MFI) and (c) Percentage of IL-1Ra-producing cells in the indicated leukocyte subpopulations. (d) PRRSV genomic copy numbers detected in the sera of the experimental pigs and (e) percentages of IL-1Ra-producing cells in the myeloid subpopulation during the observation period. PRRSV-seronegative pigs (eight pigs per group) were vaccinated with type 2 PRRSV-MLV or PBS at 0 days p.i. Subsequently, serum and heparinized whole blood samples were collected at 0, 4, 7 and 14 days p.i. *** and ** indicate significant difference at $P<0.0001$ and $P<0.001$, respectively.

are shown in Fig. 4(e). The numbers of IL-1Ra-producing cells in the myeloid subpopulation significantly increased from 4 to 7 days p.v. (Fig. 4e). Therefore, consistent with the *in vitro* findings, type 2 PRRSV enhanced IL-1Ra production in the myeloid subpopulation of the infected pigs.

HP-PRRSV induced different patterns of *IL1RA* and pro-inflammatory cytokine gene expression compared to type 2 PRRSV

HP-PRRSV exhibits different clinical outcomes and patterns of inflammatory cytokine responses compared with the typical type 2 PRRSV strain [52, 53]. In this study, the kinetics of *IL1RA* and other innate cytokine gene expression in the presence of type 2 PRRSV (01NP1) and HP-PRRSV (10PL01) were compared. In contrast to type 2 PRRSV, HP-PRRSV did not enhance *IL1RA* gene expression in the cultured MoDC (Fig. 5a). To confirm the *in vitro* findings, PRRSV-seronegative pigs were vaccinated with type 2 PRRS-MLV or intranasally inoculated with $10^{4.5}$ $TCID_{50}$ of HP-PRRSV (10PL01). HP-PRRSV (10PL01) infection resulted in high fever, depression, severe respiratory distress and pathological changes, consistent with the clinical manifestation patterns induced by HP-PRRSV [54, 55] (data not shown). Viral genomic RNA was detected in the infected

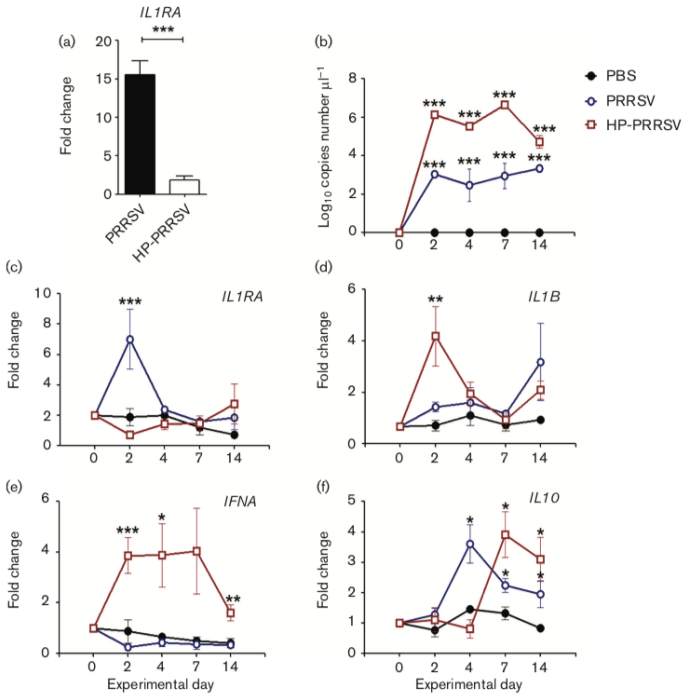

Fig. 5. HP-PRRSV induced different patterns of *IL1RA* and other cytokine gene expression. (a) Levels of *IL1RA* gene expression induced by PRRSV and HP-PRRSV. Porcine MoDCs (five pigs per group) were cultured with 0.1 m.o.i. of HP-PRRSV, 0.1 m.o.i. of type 2 PRRSV (01NP1) or MARC-145 cell lysate for 24 h. Data represent the level of *IL1RA* gene expression obtained from the treatment subtracted by that of mock-infected MoDC. The levels of (b) PRRSV genomic copy numbers, (c) *IL1RA*, (d) *IL1B*, (e) *IFNA* and (f) *IL10* gene expression in pigs of the experimental groups during the observation period. PRRSV-seronegative pigs were vaccinated with type 2 PRRSV-MLV or intranasally inoculated with HP-PRRSV, PBS (five pigs per group) at 0 days p.i. Subsequently, serum and heparinized whole blood samples were collected at 0, 2, 4, 7 and 14 days p.i. ***, ** and * indicate significant difference at $P<0.0001$, $P<0.001$ and $P<0.05$, respectively.

pigs from 2 days p.i. to the end of the observation period (14 days p.i.) (Fig. 5b). In the pigs receiving MLV, upregulation of *IL1RA* gene expression was clearly observed (Fig. 5c), while upregulation of *IL1B* and *IFNA* gene expression was not evident during the same period (Fig. 5d, e). Upregulation of *IL10* gene expression was observed later than that of *IL1RA* at 4 days p.v. (Fig. 5f). Consistent with the *in vitro* findings, HP-PRRSV infection did not upregulate *IL1RA* gene expression in the infected pigs throughout the experiment (Fig. 5c). Infection with HP-PRRSV significantly enhanced *IL1B* and *IFNA* gene expression in the infected pigs, from 2 days p.i. (Fig. 5d, e), while upregulation of *IL10* gene expression was observed from 7 days p.i. Together, our findings indicated that type 2 PRRSV and HP-PRRSV induced different patterns of *IL1RA* and other innate cytokine gene expression.

DISCUSSION

To investigate the immunomodulatory mechanism of early PRRSV infection, we identified IL-1Ra as a potential negative immunomodulatory mediator. The effects of PRRSV on induction of IL-1Ra were demonstrated, both *in vitro* and *in vivo*. Notably, both type 2 PRRSV (strain 01NP1) and type 2 PRRS-MLV were able to induce IL-1Ra production in this study. The findings supported previous opinions, indicating that the immunomodulatory properties of PRRS-MLV are similar to those of field PRRSV [50, 51]. PRRSV primarily

infects the myeloid cell population, including macrophages and dendritic cells [22, 42], which appear to be responsible for IL-1Ra production. Consistent with the findings in this study, the cells of myeloid lineage, including monocytes/macrophages, dendritic cells and neutrophils, have been reported as the major IL-1Ra producers in humans [56–59]. It should be noted that the production of IL-1Ra was observed at the very early stage following PRRSV infection, concurrent with the absence of pro-inflammatory cytokines and type I IFN gene expression, while enhanced IL-10 gene expression was observed at the later time point (Fig. 5). Although it has been indicated that PRRSV-induced IL-10 can negatively modulate the host immune responses [35, 60], the results from this study suggested that PRRSV-induced IL-1Ra production, rather than IL-10, was likely responsible for the decrease in pro-inflammatory cytokine and type I IFN production during the early phase of PRRSV infection. The findings on different patterns of IL-1Ra production following type 2 PRRSV and HP-PRRSV infections further highlighted the potential regulatory role of IL-1Ra, which could affect the clinical outcome following PRRSV infections.

IL-1Ra is known as an early inhibitory cytokine that which plays an important regulatory role in acute inflammatory response [56]. One previous report demonstrated that IL-1Ra could modulate the production of pro-inflammatory cytokines and type I IFN; IL-1Ra suppressed the production of IL-1α-induced IL-6 and IL-8 in the epithelial cells [61]. Exogenous IL-1Ra inhibited the production of IL-1β and TNF-α in LPS-stimulated monocytes [43]. In rheumatoid arthritis patients, treatment with synthetic IL-1Ra peptide drastically decreased the production of IL-1β, IL-6 and TNF-α in the affected joints [62]. In addition, it has been shown that IL-1Ra modulated IFN-α and IFN-β production in a mouse model [44, 63]. IL-1Ra also modulated the adaptive immune functions by reduction of antigen-specific T-cell activation, proliferation and polarization [64–66]. Furthermore, lack of an IL-1 receptor signalling cascade inhibited the polarization of T-helper type 2 and production of antigen-specific antibodies [66–68]. Together, the above information indicates that IL-1Ra production influences host immunocompetency.

In general, typical PRRSV infection induced weak innate cytokine production and delayed cell-mediated and humoral immunity in infected pigs [29]. It is likely that early induced and prolonged IL-1Ra production will influence the induction of innate and, subsequently, adaptive immune responses resulting in the unique immunological profiles observed following PRRSV infection. Supporting this notion, enhanced IL-1Ra production played a crucial role in suppression of IL-1/IL-8-induced inflammatory responses during an early phase of *Yersinia pestis* infection. It is believed that induction of IL-1Ra accommodated bacterial survival within the infected lungs [69]. The induction of IL-1Ra by *Aspergillus fumigatus* correlated with increased fungal burden and suppressed inflammation in the infected lungs [70]. Overexpression of IL-1Ra enhanced *Listeria monocytogenes* replication and interfered with the macrophage maturation process in infected mice [71]. In HIV-infected patients, levels of serum IL-1Ra correlated with decreased CD8 activation and enhanced viral load during the acute phase of HIV infection [72]. The biological relevance of PRRSV-induced IL-1Ra on the functions of porcine innate and adaptive immune functions is currently under investigation.

In contrast to typical PRRSV, HP-PRRSV induces strong innate cytokine production during the early phase of infection and causes severe clinical disease in infected pigs [17, 19, 20]. Our findings that HP-PRRSV enhanced high *IL1B* and *IFNA* gene expression (Fig. 5d, e) are consistent with the above reports. To date, the reasons for variation in the pathogenesis of HP-PRRSV are not clearly understood [73, 74]. In contrast to the typical type 2 PRRSV, HP-PRRSV did not induce IL-1Ra, either *in vitro* or *in vivo*. Our findings suggest that inability to induce IL-1Ra production could be one of the underlined mechanisms, resulting in uncontrolled production of innate cytokines and severe clinical outcome following HP-PRRSV infection. Interestingly, mice lacking IL-1Ra exhibited enhanced production of pro-inflammatory cytokines, including IL-1, IL-6 and IL-17, and increased mortality in the subsequent Ebola virus infection [75]. The role of IL-1Ra in the immunopathogenesis of HP-PRRSV should be further explored.

It should be pointed out that different PRRSV strains possess different immunomodulatory properties – in particular, induction of IL-10 production and viral-specific Treg [39, 76, 77]. In this study, the potential of other PRRSV strains, including type 1 (EU) PRRSV, and other type 2 PRRSV strains or other PRRSV-MLV, to induce IL-1Ra production was not explored due to limited availability of the viruses. It would be interesting to further investigate whether the ability to induce IL-1Ra production is conserved among the PRRSV strains. In addition, the precise molecular mechanism responsible for PRRSV-induced IL-1Ra production is not known. This information will be crucial for better understanding of the immunopathogenesis of PRRSV.

In conclusion, this study identified IL-1Ra as a negative immunomodulatory mediator during the early phase of PRRSV infection. In addition, type 2 PRRSV and HP-PRRSV exhibited variation in their capability in regard to induction of IL-1Ra. These findings could partly explain the differences in immunopathogenesis and clinical outcomes following PRRSV infection.

METHODS

Viruses and cells

Type 2 PRRSV, strain 01NP1 [78], HP-PRRSV, strain 10PL01 [79], classical swine fever virus, swine influenza virus H1N1 (AT/swine/Thailand/CU-PS73/2010) and H3N2 (A/swine/Thailand/CU-CB8.4/2007) were kindly provided by the Chulalongkorn University Veterinary

Diagnostic Laboratory (CU-VDL; Bangkok, Thailand). PRRSV was propagated and titrated in the MARC-145 cell line (CU-VDL) at 10^6 $TCID_{50}$ ml^{-1}. CSFV and influenza virus were propagated to 10^6 $TCID_{50}$ ml^{-1} in the SK-6 (CU-VDL) and MDCK cell lines (CU-VDL), respectively. Mock-infected cell lysates were prepared from MARC-145 (for PRRSV), SK-6 (for CSFV) and MDCK (for influenza virus) cell lines. All viruses and mock-infected cell lysates were stored at −80 °C until needed.

Antibodies and secondary conjugates

Anti-swine CD3-FITC mAb (BB23-8E6, IgG2b) conjugate and biotinylated anti-swine SWC3 mAb (74-22-15, IgG1) were purchased from SouthernBiotech. Anti-swine sIg (H+L)-PE polyclonal Ab (15H6, IgG1) was purchased from AbSerotec. Anti-swine IL-1Ra mAb (114801, IgG2a) and the isotype controls were purchased from R&D Systems. Streptavidin-APC and goat anti-mouse IgG2a-PE/cy7 were purchased from BioLegend. Anti-PRRSV mAb (SDOW-17, RTI, SD, US) was provided by CU-VDL.

Animals and animal experiments

Eight-week-old, crossbred, PRRSV-seronegative pigs (five pigs per group) were obtained from a commercial farm in Kanchanaburi Province, Thailand. The pigs were randomly grouped and housed throughout the experiment at the animal facility of the Faculty of Veterinary Medicine Kasetsart University, Kumpangsan campus the pigs were vaccinated with 2 ml PBS, 2 ml of a commercial type 2 MLV (Ingelvac PRRS Boehringer Ingelheim Vetmedica) or intranasally inoculated with 2 ml of $10^{4.5}$ $TCID_{50}$ ml^{-1} HP-PRRSV (10PL01). Heparinized whole blood and serum samples were collected at 0, 2, 4, 7 and 14 days p.i. The blood samples were subjected for PBMC isolation and further immunofluorescent staining. Serum samples were subjected for detection of PRRSV using quantitative reverse transcriptase PCR (see below). The samples were collected from the experimental pigs specifically for the purposes of this study.

Isolation of porcine PBMCs and PBLs, generation of MoDCs and *in vitro* activation assays

PBMCs were isolated from heparinized blood samples by gradient centrifugation, using LymphoSep™ separation medium (MP Biomedicals) according to the manufacturer's protocol. For the generation of MoDC, the PBMCs were resuspended at 5×10^6 cells ml^{-1} in Iscove's modified Dulbecco's medium (Gibco) and plated on a 24-well plate (Corning-Costar) for 2 h. Non-adherent cells, containing 74.7±8.35 % CD3 and 16.1±3.76 % sIg cells, referred to as PBLs, were collected and stored in liquid nitrogen until needed. The remaining adherent cells, referred to as monocytes, were cultured in 1 ml of RPMI medium containing advanced RPMI (Gibco), 10 % FBS (Gibco), 2 mM L-glutamine (Gibco), antibiotic/antimycotic solution (Gibco), 25 mM HEPES (Gibco) and 50 µM β-mercaptoethanol (Sigma Chemical), supplemented with 10 ng ml^{-1} porcine recombinant IL-4 (R&D Systems) and 25 ng ml^{-1} porcine recombinant GM-CSF (R&D Systems), referred to as DC medium, for 7 days, with 50 % replacement with fresh DC medium every 2 days. For *in vitro* activation, the porcine leukocyte populations (PBMC, PBL and MoDC) were cultured with 0.1 m.o.i. of the indicated viruses or the relevant mock-infected cell lysates on a 24-well plate at 37 °C in a 5 % CO_2 incubator for 24 or 48 h, as indicated in the legends.

Microarray analysis

MoDCs were cultured with 0.1 m.o.i. of type 2 PRRSV (01NP1) or MARC-145 cell lysate on a 24-well plate at 37 °C in a 5 % CO_2 incubator for 24 h. Total mRNA was purified using a commercial RNA extraction kit (Qiagen) according to the manufacturer's instructions. Samples were hybridized onto Porcine_GXP_4×44K (two-colour microarray-based gene expression analysis). The arrays were scanned with an Agilent scanner system, and data were collected using an Agilent Feature Extraction Software v10.7. Data were normalized using GeneSpring GX 11.5 software. The fold values were provided in terms of log base 2 when comparing the two groups: PRRSV (P) and mock-infected MoDC (M).

All microarray data have been approved and deposited in the Gene Expression Omnibus under accession number GSE86182.

Quantitative reverse transcriptase PCR detection of PRRSV RNA

Viral RNAs were extracted from the serum samples and lung tissues (approximately 1×1×0.5 cm) using an RNA extraction kit (NucleoSpin RNA virus kit; MACHEREY-NAGEL) according to the manufacturer's protocol. Quantification of PRRSV RNA was performed using TaqMan probe-based real-time reverse transcriptase PCR as previously described [80]. The sequences of the probe and primers are as follows: US-PRRSV-specific probe (5′ FAM-TCC-CGG-TCC-CTT-GCC-TCT-GGA-TAMRA 3′), ORF7-US forward primer (5′ AAA-TGI-GGC-TTC-TCI-GGI-TTTT 3′) and ORF7-US reverse primer (5′ AAA-TGI-GGC-TTC-TCIG-GIT-TTT 3′). The amplification was carried out in a 25 µl reaction containing SuperScript III One-Step RT-PCR kit (Thermo Fisher Scientific) 1× reaction mix, 0.4 mM of each primer and probe, 0.5 µl (100 U) of SuperScript III RT/Platinum Taq Mix and 0.5 µl (20 pmol) of viral RNA.

qPCR for detection of porcine cytokine gene expression

Total mRNAs were extracted from the leukocyte subpopulations (PBMC, PBL and MoDC) using a commercial RNA extraction kit (Biotechrabbit, Germany) according to the manufacturer's instructions. The extracted mRNAs were quantified using NanoDrop (Thermo Scientific) and converted to cDNA using a commercial cDNA synthesis kit (Invitrogen). The levels of cytokine gene expression were determined using SYBR Green-based real-time PCR. The qPCR for detection of porcine *IL1RA*, *IL1B*, *IFNA*, *IL10* and *GAPDH* gene expression was performed using

Table 1. Oligonucleotide sequences of primers used for qPCR

Gene	NCBI accession no.	Primer sequence (5′→3′)	Product size (bp)	Reference
IL1RA	AY577820.1	F: CTT-CAC-CTT-CAT-CCG-CTC R: TGG-TCC-TGC-TGG-AAG-TAG	268	–
IL1B	NM_214055.1	F: AAC-GTG-CAA-TGA-TGA-CTT-TG R: CAC-TTC-TCT-CTT-CAA-GTC-CC	292	–
IL6	JQ839263.1	F: AGA-ACT-CAT-TAA-GTA-CAT-CCT-CG R: AGA-TTG-GAA-GCA-TCC-GTC	180	–
IFNA	XM_003480507.3	F: CTG-GAG-GAG-GAC-TCC-AT R: GAG-TCT-GTC-TTG-CAG-GTT	268	–
IL10	HQ236499.1	F: AGC-CAG-CAT-TAA-GTC-TGA-GAA R: CCT-CTC-TTG-GAG-CTT-GCT-AA	394	[81]
GAPDH	XM_005658673.2	F: AAG-TGG-ACA-TTG-TCG-CCA-TC R: TCA-CAA-ACA-TGG-GGG-CAT-C	318	[81]

the primer sets described in Table 1. The qPCR was carried out in a 20 µl reaction and consisted of 2 µl (20 ng) of the cDNA template, 0.5 µl (20 pmol) of each specific primer, 7 µl of sterile water and 10 µl of SYBR Green master mix (Biotechrabbit). qPCR was carried out using a Rotor-Gene RG-3000 (Corbett Research). The amplification reaction consisted of initial denaturation at 95 °C for 15 min, followed by 40 cycles of denaturation at 94 °C for 20 s, annealing at 55 °C for 30 s and extension at 72 °C for 30 s. The specificity of the amplicon was verified by melting curve analyses. The C_t values of gene expression were normalized against the housekeeping gene *GAPDH*. Differences in C_t values between the treatment groups were analysed by the formula $2^{-\Delta\Delta C_t}$.

Detection of IL-1Ra in the cultured supernatants

Following *in vitro* activation, supernatants obtained from the cell culture were collected and stored at −80 °C until used. Determination of IL-1Ra protein was performed using a commercial ELISA kit (CUSABIO), according to the manufacturer's protocol.

Immunofluorescent staining and flow cytometric analyses

The cells of interest were harvested, washed once with Ca-, Mg-free PBS (PBSA) and then resuspended in PBSA supplemented with 0.5 % BSA and 0.1 % sodium azide, referred to as the FACS buffer. The cells were distributed ion a 96-well plate (Corning-Costar) at the concentration of 2×10^6 cells per well and pelleted by centrifugation at 500 ***g*** for 5 min. Immunofluorescent staining of the surface molecules, CD3, SWC3 and sIg, was performed by addition of 1 : 50 of anti-CD3-FITC, 1 : 100 of biotinylated anti-SWC3 and 1 : 100 of anti-sIg (H+L)-PE antibodies, diluted in FACS buffer and followed by incubation at 4 °C in the dark for 40 min. The stained cells were then washed twice with PBSA. For secondary staining of SWC3, 1 : 500 of streptavidin-APC diluted in FACS buffer was added to the cells followed by incubation at 4 °C in the dark for 30 min.

For intracellular staining, the protein transport inhibitor monensin (GolgiStop; BD Biosciences), was added to the culture 12 h prior to harvesting. Following harvesting and staining of the surface molecules, the cells were then fixed and permeabilized with 100 µl per well of 50 % reagent A (Leucoperm, Serotec), then diluted in FACS buffer at room temperature in the dark for 30 min. For primary staining, 1 : 100 of anti-IL-1Ra (IgG2a), diluted in reagent B (Leucoperm), was added to the cells and further incubated at 4 °C in the dark for 45 min. For the secondary staining required for staining of IL-1Ra, 1 : 100 of goat anti-mouse IgG2a-PE/cy7, diluted in FACS buffer, was added to the cells followed by incubation at 4 °C in the dark for 30 min. As a final step, the stained cells were resuspended in 2 % formaldehyde in PBSA and kept at 4 °C until flow cytometric analyses were performed.

Similar cells stained with isotype controls were included and used as the background cut-off in this study. Fluorescent minus one staining samples were also performed during the establishment and validation of the assay. Flow cytometric analyses were performed using a Beckman Coulter FC 500 MPL.

Statistical analyses

Data were analysed using Student *t*-test or ANOVA, followed by Tukey's multiple comparison tests all statistical analyses were performed using GraphPad Prism for Windows (GraphPad Software Incorporated).

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Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement

The animal care and use protocols for this study adhered to the Ethical Principles and Guidelines for the Use of Animals, National Research Council of Thailand, and the Guide for the Care and Use of Laboratory Animals, National Research Council, USA. All methods and animal studies were conducted under the approval of the Chulalongkorn University Animal Care and Use Committee, Chulalongkorn University (animal use protocol no. 1631029).

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VITA

Mr. Teerawut Nedumpun was born on May 17th, 1988 in Thailand. In 2006, I enrolled in the Degree of Doctor of Veterinary Medicine in the Faculty of Veterinary Science, Chulalongkorn University, Thailand. After graduation with the first-class honors, I started in Interdisciplinary program in Medical Microbiology, Graduate School, Chulalongkorn University in academic year 2012.

During study in the Ph.D. program, I have obtained the scholarship from the Royal Golden Jubilee Ph.D. Program Thailand Research Fund, Chulalongkorn University Graduate Scholarship to commemorate the 72nd anniversary of His Majesty King Bhumibol Adulyadej and the 90th Anniversary of Chulalongkorn University Ratchadaphiseksomphot Endowment Fund.

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