ผลของการใช้ดีเอ็นเอวัคซีนร่วมกับวัคซีนพีอาร์อาร์เอสชนิดเชื้อเป็น ต่อการปรับเปลี่ยนการตอบสนองทางภูมิคุ้มกันต่อเชื้อพีอาร์อาร์เอสชนิดสายพันธุ์รุนแรงในสุกร

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คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
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EFFECTS OF DNA-MLV PRIME BOOST IMMUNIZATION ON MODULATION OF PRRSV-SPECIFIC IMMUNE RESPONSES IN HP-PRRSV CHALLENGED PIGS

Mr. Chaitawat Sirisereewan

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By	Mr. Chaitawat Sirisereewan
Field of Study	Veterinary Pathobiology
Thesis Advisor	Professor Sanipa Suradhat, D.V.M., Ph.D., DTBVP.
Thesis Co-Advisor	Professor Roongroje Thanawongnuwech, D.V.M., M.Sc., Ph.D., DTBVP.

Accepted by the Faculty of Veterinary Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

.....Dean of the Faculty of Veterinary Science

(Professor Roongroje Thanawongnuwech, D.V.M., M.Sc., Ph.D., DTBVP.)

THESIS COMMITTEE

.....Chairman

(Associate Professor Theerayuth Kaewamatawong, D.V.M., Ph.D., DTBVP.)

.....Thesis Advisor

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

.....Thesis Co-Advisor

(Professor Roongroje Thanawongnuwech, D.V.M., M.Sc., Ph.D., DTBVP.)

.....Examiner

(Assistant Professor Sawang Kesdangsakonwut, D.V.M., M.Sc., Ph.D., DTBVP.)

.....External Examiner

(Assistant Professor Pariwat Poolperm, D.V.M., Ph.D., DTBVM.)

ชัยธวัช ศิริเสรีวรรณ : ผลของการใช้ดีเอ็นเอวัคซีนร่วมกับวัคซีนพีอาร์อาร์เอสชนิดเชื้อเป็น ต่อการปรับเปลี่ยนการตอบสนองทางภูมิคุ้มกันต่อเชื้อพีอาร์อาร์เอสชนิดสายพันธุ์รุนแรงในสุกร (EFFECTS OF DNA-MLV PRIME BOOST IMMUNIZATION ON MODULATION OF PRRSV-SPECIFIC IMMUNE RESPONSES IN HP-PRRSV CHALLENGED PIGS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ศ. สพ.ญ. ดร. สันนิภา สุรทัตต์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ศ. น.สพ. ดร. รุ่งโรจน์ ธนาวงษ์นุเวช, 65 หน้า.

ไวรัสพีอาร์อาร์เอส จัดเป็นไวรัสก่อโรคที่สำคัญในสุกร ที่ก่อให้เกิดความเสียหายอย่างรุนแรงต่ออุตสาหกรรมการเลี้ยงสุกรทั่วโลก ไวรัสพีอาร์อาร์เอสเป็นไวรัสที่มีความหลากหลายทางพันธุกรรมสูง จึงเหนี่ยวนำให้เกิดกลายพันธุ์ของไวรัสจนเกิดเป็นไวรัสพีอาร์อาร์เอสชนิดสายพันธุ์รุนแรง การระบาดของไวรัสพีอาร์อาร์เอสชนิดสายพันธุ์รุนแรงเกิดขึ้นครั้งแรกในประเทศจีน ในปี 2549 หลังจากนั้นไวรัสดังกล่าวได้แพร่กระจายเข้ามายังหลายประเทศในภูมิภาคเอเชียตะวันออกเฉียงใต้ ปัจจุบันการใช้วัคซีนโรคพีอาร์อาร์เอสชนิดเชื้อเป็นไม่สามารถให้ผลในการป้องกันการติดเชื้อไวรัสพีอาร์อาร์เอสชนิดสายพันธุ์รุนแรงได้อย่างสมบูรณ์ ทั้งยังส่งผลกระทบในเชิงลบต่อการทำงานของระบบภูมิคุ้มกันในสุกร เป็นที่น่าสนใจว่าการใช้ดีเอ็นเอวัคซีนที่มียีนที่สร้างโปรตีนนิวคลิโอแคปสิดบางส่วนของเชื้อพีอาร์อาร์เอส ร่วมกับวัคซีนโรคพีอาร์อาร์เอสชนิดเชื้อเป็นสามารถช่วยกระตุ้นการตอบสนองทางภูมิคุ้มได้ดียิ่งขึ้น เพื่อศึกษาประสิทธิภาพของการใช้ดีเอ็นเอวัคซีนร่วมกับวัคซีนโรคพีอาร์อาร์เอสชนิดเชื้อเป็น ต่อการติดเชื้อไวรัสพีอาร์อาร์เอสชนิดสายพันธุ์รุนแรง ทำการแบ่งสุกรทดลองปลอดเชื้อไวรัสพีอาร์อาร์เอสออกเป็นจำนวน 5 กลุ่มการศึกษา เมื่อสุกรอายุประมาณ 2 สัปดาห์ (ในวันที่ -14; D-14) สุกรทดลองกลุ่ม DNA-MLV และ MLV จะได้รับดีเอ็นเอวัคซีน (pORF7t) และสารละลายพีบีเอส (PBS) ตามลำดับ และให้วัคซีนโรคพีอาร์อาร์เอสชนิดเชื้อเป็น (MLV) ในวันที่ 0 สำหรับสุกรทดลองกลุ่มอื่นๆ ประกอบด้วย สุกรทดลองกลุ่ม DNA ซึ่งได้รับการจะได้รับดีเอ็นเอวัคซีน (pORF7t) ในวันที่ -14 ตามด้วยการให้สารละลายพีบีเอส (PBS) ในวันที่ 0 สุกรกลุ่ม PBS จะได้รับสารละลายพีบีเอส (PBS) ในวันที่ 0 และกลุ่มควบคุมลบ (negative) เมื่อสุกรอายุ 6 สัปดาห์ ทำการฉีดพิษทับด้วยเชื้อไวรัสพีอาร์อาร์เอสชนิดสายพันธุ์รุนแรงแก่สุกรทดลองทุกกลุ่มยกเว้นกลุ่มควบคุมลบ ผลการศึกษาพบว่ากลุ่มสุกรทดลองในกลุ่ม DNA-MLV และ MLV แสดงอาการทางคลินิกที่น้อยกว่า และสามารถกำจัดเชื้อไวรัสได้เร็วยิ่งขึ้น ภายหลังการฉีดพิษทับ พบว่าสุกรทดลองกลุ่ม DNA-MLV มีการตอบสนองทางภูมิคุ้มกันที่ดีกว่ากลุ่ม MLV โดยตรวจพบการระดับนิวทรัลไลซิ่งแอนติบอดี (neutralizing antibody) ต่อเชื้อไวรัสพีอาร์อาร์เอสชนิดสายพันธุ์รุนแรง และการตอบสนองทางภูมิคุ้มกันชนิดเซลล์ต่อเชื้อไวรัสพีอาร์อาร์เอส โดยการสร้างอินเตอร์ฟิรอนแกมม่า (IFN-g) ได้ดียิ่งขึ้น อีกทั้งยังสามารถลดการตอบสนองทางภูมิคุ้มกันในเชิงลบ โดยลดการการสร้างอินเตอร์ลิวคิน 10 (Interleukin-10;IL-10) และ PRRSV-specific Treg ได้ อย่างไรก็ตาม การให้วัคซีน DNA-MLV ในรูปแบบไพรม์-บูส (prime-boost) และการให้วัคซีนเชื้อเป็นเพียงอย่างเดียวไม่สามารถให้ผลการป้องกันการติดเชื้อไวรัสพีอาร์อาร์เอสชนิดสายพันธุ์รุนแรงได้อย่างสมบูรณ์ ผลจากการศึกษานี้ สรุปว่า แม้ว่าการให้วัคซีนในรูปแบบไพรม์-บูส จะช่วยปรับเปลี่ยนการตอบสนองทางภูมิคุ้มกันต่อเชื้อพีอาร์อาร์เอสได้ดีขึ้น แต่ไม่สามารถป้องกันการติดเชื้อย่างสมบูรณ์ได้ การศึกษานี้ชี้ให้เห็นว่า การใช้ดีเอ็นเอวัคซีนร่วมกับวัคซีนในรูปแบบอื่นๆ (prime-boost strategy) สามารถกระตุ้นการสร้างภูมิคุ้มกันที่มีประสิทธิภาพสูงขึ้น ซึ่งอาจนำไปสู่การป้องกันการติดเชื้อไวรัสพีอาร์อาร์เอสอย่างสมบูรณ์ได้

ภาควิชา	พยาธิวิทยา	ลายมือชื่อนิสิต	----------
สาขาวิชา	พยาธิชีววิทยาทางสัตวแพทย์	ลายมือชื่อ อ.ที่ปรึกษาหลัก	----------
ปีการศึกษา	2559	ลายมือชื่อ อ.ที่ปรึกษาร่วม	----------

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CHAITAWAT SIRISEREEWAN: EFFECTS OF DNA-MLV PRIME BOOST IMMUNIZATION ON MODULATION OF PRRSV-SPECIFIC IMMUNE RESPONSES IN HP-PRRSV CHALLENGED PIGS. ADVISOR: PROF. SANIPA SURADHAT, D.V.M., Ph.D., DTBVP., CO-ADVISOR: PROF. ROONGROJE THANAWONGNUWECH, D.V.M., M.Sc., Ph.D., DTBVP., 65 pp.

Porcine reproductive and respiratory syndrome virus (PRRSV) has been recognized as the important swine pathogen that threaten swine industry globally. The quasispesies character of PRRSV causes genetic variation, leading to the emergence of the new variant type 2 PRRSV, known as highly pathogenic PRRSV (HP-PRRSV). HP-PRRSV first emerged in China in 2006 and then spread to circulate in the Southeast Asia region. Currently, commercially available modified live PRRS vaccines (MLV) are not able to provide complete protection against the HP-PRRSV, and have been reported to induce negative immunomodulatory effects in vaccinated pigs. Interestingly, a novel DNA vaccine was developed and successfully used to improve PRRSV-specific immune responses following MLV vaccination. To investigate the efficacy of a heterologous DNA-MLV prime-boost immunization against the HP-PRRSV infection, an experimental vaccinated-challenged study was conducted. Two-week-old, PRRSV-seronegative, crossbred pigs (5-8 pigs/group) were allocated into 5 groups. At day-14 (D-14), the treatment group (DNA-MLV) was immunized with a DNA vaccine encoding PRRSV-truncated nucleocapsid protein (pORF7t), followed by a commercial modified live, type 2 PRRS vaccine (MLV) at D0. The other groups included the group that received PBS at D-14 followed by MLV at D0 (MLV), pORF7t at D-14 (DNA), PBS at D0 (PBS) and the negative control group. At D42, all groups, except the negative control group, were challenged with HP-PRRSV (strain 10PL1). The results demonstrated that pigs that received MLV, regardless of the DNA priming, exhibited less clinical signs and faster viral clearance. Following HP-PRRSV challenge, the DNA-MLV group exhibited improved PRRSV-specific immunity, as observed by increased neutralizing antibody titers and PRRSV-specific IFN-ɡ production, and reduced IL-10 and PRRSV-specific Treɡ productions. However, neither the prime-boost immunization nor the MLV was able to induce complete clinical protection against HP-PRRSV infection. In conclusion, improved immunological responses, but not complete clinical protection, were achieved by DNA-MLV prime-boost immunization. This study highlights the potential use of heterologous prime-boost vaccination regimen, where DNA can be incorporated with other vaccine candidates, for improving anti-PRRSV immunity that may eventually lead induction of complete PRRSV protection.

Department:	Veterinary Pathology	Student's Signature .....
Field of Study:	Veterinary Pathobiology	Advisor's Signature .....
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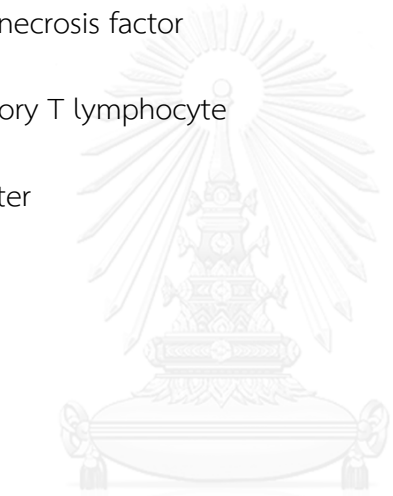
LIST OF ABBREVIATIONS

ADE	antibody-dependent enhancement
BSA	bovine serum albumin
CU-VDL	Veterinary Diagnosis Laboratory, Chulalongkorn University
dpi	day post infection
ELISA	enzyme-linked immunosorbent assay
EU	European
FITC	fluorescein isothiocyanate
FoxP3	forkhead box P3
G	gram (s)
GP	glycoprotein
H&E	hematoxylin and eosin
HP-PRRSV	highly-pathogenic porcine reproductive and respiratory syndrome virus
HS	heparan sulfate
IFN	interferon
IL	interleukin
IPMA	indirect immunoperoxidase assay
Kb	kilobase pair
KV	killed vaccine

mAb	monoclonal antibody
MEM	minimum essential media
ml	millilitre (s)
MLV	modified live vaccine
mM	millimolar
mm^3	millimetre (s)
m.o.i.	multiplicity of infection
NA	North American
NAb	neutralizing antibody
N-protein	nucleocapsid protein
NSP	non-structural protein
ORF	open reading frame
ORF7	nucleocapsid protein
ORF7t	truncate nucleocapsid protein
PAM	pulmonary alveolar macrophage
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PRDC	porcine respiratory disease complex
PRRSV	porcine reproductive and respiratory syndrome virus
qPCR	quantitative polymerase chain reaction

RPMI	Roswell Park Memorial Institute medium
RT-PCR	reverse transcriptase polymerase chain reaction
SEM	standard error of the means
SN	serum neutralization
Sn	sialoadhesin
$TCID_{50}$	tissue culture infectious dose 50
TNF	tumor necrosis factor
Treg	regulatory T lymphocyte
μl	microliter



CHAPTER 1

INTRODUCTION

1.1 Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) is one of the most important swine pathogens that significantly causes major economic impact on swine industry worldwide. PRRSV infection is characterized by reproductive failure in breeding herd, respiratory disorders in nursery to finishing pigs with predisposing to secondary infections, known as porcine respiratory disease complex (PRDC) (Lunney et al., 2016). Several studies suggested that PRRSV-induced negative immunomodulatory effects resulted in poor anti-viral immune responses, and immunosuppression in infected pigs (Lopez and Osorio, 2004; Mateu and Diaz, 2008; Kimman et al., 2009; Cecere et al., 2012). Thus, controlling PRRSV infection is essential for enhancing pig growth performance and economic profit. Currently, vaccination against PRRSV, both modified live PRRS vaccines (MLVs) and killed vaccines (KVs), had been licensed (Kimman et al., 2009; Charerntantanakul, 2012). However, these vaccines do not provide complete protection against field strains or heterologous infections (Charerntantanakul, 2012; Renukaradhya et al., 2015). In addition, similar to the natural infection, MLV could also induce negative immunomodulatory effects, leading to reduce vaccine efficacy (Thanawongnuwech and Suradhat, 2010; LeRoith et al., 2011; Suradhat et al., 2016).

Recently, a new variant type 2 PRRSV, known as highly pathogenic PRRSV (HP-PRRSV), emerged in China, causing severe clinical outcomes and high mortality in infected pigs (Tian et al., 2007). Since the initial outbreak, HP-PRRSV rapidly spread to other countries and became the dominant virus circulated in the region, including Thailand (Nilubol et al., 2012; Jantafong et al., 2015). Previous studies from China indicated that HP-PRRSV derived MLVs provided full protection against HP-PRRSV infection (Leng et al., 2012; Yu et al., 2015). However, the vaccines were not licensed in other countries and reversion of HP-PRRSV MLVs should be concerned. The current commercially available MLVs only provided partial protection against the HP-PRRSV challenges (Lager et al., 2014; Do et al., 2015). Recently, a novel DNA vaccine (pORF7t), has been developed. The vaccine was designed to modulate PRRSV-specific immune responses by reducing PRRSV-induced immunomodulatory activities (Suradhat et al., 2015). Priming with DNA vaccine could reduce MLV-induced negative immunomodulatory effects, both IL-10 and Treg, and also enhance PRRSV-specific cell-mediated immunity in the immunized pigs (Suradhat et al., 2016). Thus, the heterologous DNA-MLV, prime-boost immunization may be useful for controlling PRRSV infection in heavily infected areas or areas with PRRSV circulations.

We hypothesized that the DNA-MLV prime-boost immunization should enhance MLV-induced, PRRSV-specific immunity against the HP-PRRSV infection,

leading to better disease protection. The information obtained from this study will also be useful for developing PRRSV prevention and control strategy in the future.

1.2 Objectives

1. To determine the effect of the DNA-MLV prime-boost immunization on PRRSV-specific immune responses in the vaccinated, HP-PRRSV challenged model

2. To evaluate the efficacy of the DNA-MLV prime -immunization against the HP-PRRSV challenge.

CHAPTERS 2

LITERATURE REVIEW

2.1 Disease and etiology

Porcine reproductive and respiratory syndrome (PRRS) is deleterious viral disease causing significant economic impacts on pig production globally. The etiologic virus, porcine reproductive and respiratory syndrome virus (PRRSV), is an enveloped positive-strand RNA virus, and belongs to the family *Arteriviridae*, genus *Arterivirus* (Snijder and Meulenberg, 1998). PRRSV genome is approximately 15 kb in size, contains 11 open reading frames (ORFs) encoding for 14 non-structural proteins (NSP) and 8 structural proteins as shown in Figure 1. (Lunney et al., 2016). PRRSV is currently categorized into two genotypes namely; type 1 and type 2 PRRSV which are also known as European PRRSV and North America PRRSV (NA), respectively. The impact of PRRSV infection includes reproductive failure in breeding herds and respiratory disorders in nursery to finishing pigs, possibly by impairment of pulmonary defense mechanisms, leading to secondary infections, as known as porcine respiratory disease complex (PRDC) (Lunney et al., 2016).

The cellular tropism of PRRSV is the cells of the monocytic lineage, especially pulmonary alveolar macrophages (PAMs). PRRSV entry into the susceptible host cells is usually mediated through binding of the viral-specific receptors, such as CD163,

CD151, heparin sulfate (HS), sialoadhesin (Sn), and vimentin (Shi et al., 2015). The receptor-mediated entry process facilitates internalization and viral replication within the target cells (Shi et al., 2015; Zhang and Yoo, 2015). Besides of the monocytic lineages, several permissive cell lines, including CL2621, SJPL, MA-104 and MARC-145, have been used for viral propagation and *in vitro* studies of PRRSV (Zhang and Yoo, 2015).

The quasispecies characteristics, including antigenic and genetic variation, of PRRSV have been well demonstrated in both genotypes (Goldberg et al., 2003). In 2006, a highly pathogenic PRRSV (HP-PRRSV) of atypical type 2 PRRSV with deletion of 30 amino acids in the NSP2 gene, emerged in China and affected more than one million pigs. HP-PRRSV infection resulted in high morbidity and mortality in the infected pigs (Tian et al., 2007). Following the first outbreak, HP-PRRSV spread to Southeast Asian countries (Do et al., 2015; Jantafong et al., 2015). In 2010, the first outbreak of HP-PRRSV was reported in Thailand (Nilubol et al., 2012) and then became the dominant circulating PRRSV strain in Thailand (Jantafong et al., 2015). In addition to the type 2 genotype, type 1 HP-PRRSV was also reported in East Europe and designated as “Lena” strain (Karniychuk et al., 2010). The Lena strain of PRRSV was grouped in the new East European subtype 3 with 29 amino acids deletions in the NSP2 gene (Van Doorsselaere et al., 2012).

Based from phylogenetic analyses of more than 8500 ORF5 sequences from type 2 PRRSV. Type 2 PRRSV can be classified into 9 lineages with more than 10% of

interlineage genetic differences (Shi et al., 2010). Strain VR-2332, the prototype of type 2 PRRSV in the lineage 5 and had been reported in USA, Denmark, China, Korea and Thailand. Several isolates of HP-PRRSV were classified in the lineage 8, sublineage 8.7 (Shi et al., 2010; Jantafong et al., 2015). The HP-PRRSV might originate from the CH-1a-like PRRSV which was in lineage 8 (An et al., 2010). The commercial MLVs were derived from several lineages, including lineage 5 (Ingelvac PRRS MLV, Boehringer Ingelheim Vetmedica Inc.), lineage 7 (PrimePac PRRS MLV, MSD Animal Health) and lineage 9 (Ingelvac-ATP PRRS MLV, Boehringer Ingelheim Vetmedica Inc.) (Shi et al., 2010). Recently, a new type 2 PRRSV vaccine (Fostera^{TM} PRRS MLV, Zoetis), derived from lineage 8, has been introduced to the Asian markets (Do et al., 2015).

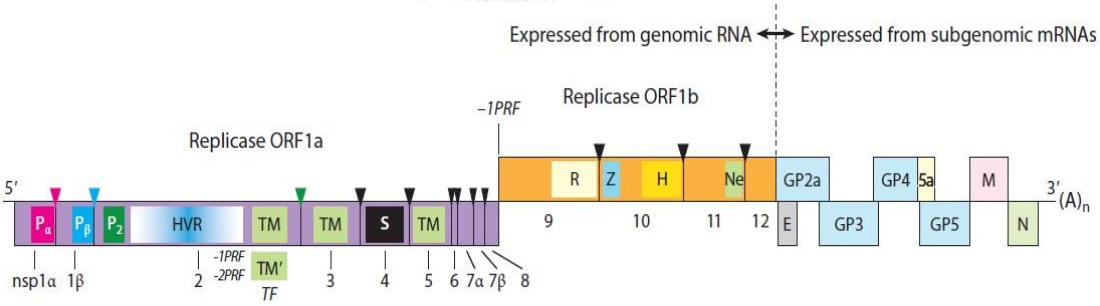


Figure 1 PRRSV genome structure; ORF1a, ORF1a-TF and ORF1b regions are translated into two large nonstructural polyproteins, particularly the replicase-associated polyproteins. ORFs 2-7 are encoded for eight structural proteins, including minor envelope proteins (GP2a, GP3, GP4, E, and ORF5a), major envelope proteins (GP5 and M), and nucleocapsid protein (N) (Lunney et al., 2016).

2.2 Transmission

Several risk factors including animal movement, biosecurity level, exposure from PRRSV-infected neighbor herds, semen, herd size, pig density and herd density have been identified as risk factors contributing to PRRSV infection (Mortensen et al., 2002). In general, the PRRSV transmission occurs both horizontally (Bierk et al., 2001) and vertically (Christianson et al., 1992).

With regard to horizontal transmission, direct contact is the most common form of PRRSV transmission, as infected pigs can shed the virus via several routes, including saliva, semen, blood, aerosols, feces, milk and colostrum as shown in Figure 2 (Cho and Dee, 2006). Virus-contaminated saliva can be easily transmitted during commingled with other penmates (Wills et al., 1997). Moreover, contaminated semen plays a significant role in PRRSV transmission through artificial insemination in breeding herds (Yaeger et al., 1993; Corzo et al., 2010). In addition, indirect transmission through contaminated fomites, transport vehicles, carrier insects, avian and non-porcine mammalian species, and aerosols, have been reported (Cho and Dee, 2006).

For vertical transmission, PRRSV can spread from sow to fetus during the late-term of gestation, as high numbers of PRRSV-specific receptors, CD163 and Sn, in the endometrium and placenta were present during the late gestation. Vertical transmission leads to fetal death, late-term abortion, mummification, early farrowing, and weak-born piglets (Karniychuk and Nauwynck, 2013). Persistent infection has been

observed for up to 150 days in post-natal infection (Allende et al., 2000) and up to 210 days in congenital infection (Cho and Dee, 2006).

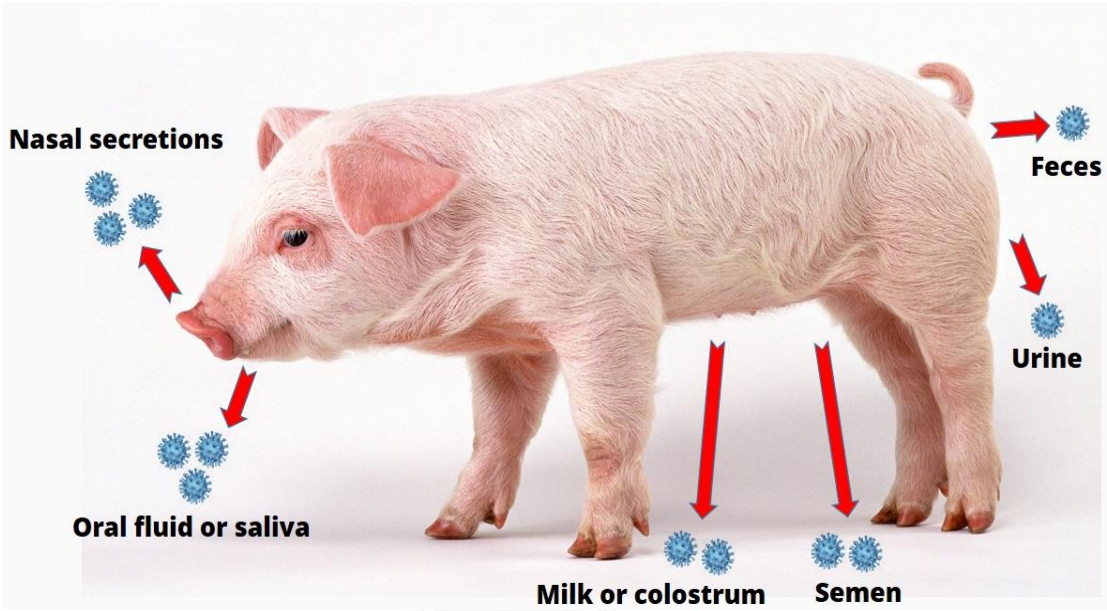


Figure 2 Main routes of PRRSV shedding in infected pigs
(Adapted from: *www.prrscontrol.com*)

2.3 Immunobiology of PRRSV

2.3.1 Immunological responses of PRRSV

2.3.1.1 Innate immune response of PRRSV

Proper innate immune response is essential for preventing viral invasion and replication, as well as inducing adaptive immune responses for complete viral clearance (Koyama et al., 2008). In contrast to other viral diseases, PRRSV infection failed to elicit several pro-inflammatory cytokines, including Interleukin (IL)-1, tumor

necrosis factor (TNF)-α and interferon (IFN)-α, during an early phase of infection (Van Reeth et al., 1999; Van Reeth and Nauwynck, 2000). Suppression of type I IFN, both IFN-α and IFN-β, following PRRSV infection had been reported (Miller et al., 2004; Loving et al., 2007). As type I IFNs inhibit PRRSV replication (Albina et al., 1998; Le Bon et al., 2001) and are necessary for induction of PRRSV-specific IFN-γ producing cells (Kadowski et al., 2000; Royaee et al., 2004). Inhibition of type I IFN contributed to poor viral clearance. Notably, the HP-PRRSV-infected pigs exhibited stronger up-regulation of pro-inflammatory cytokines, including IL-1, IL-6, IFN-α and TNF-α, than the low-virulent PRRSV strains. These cytokines were associated with severe clinical outcomes (Liu et al., 2010; Guo et al., 2013; Zhang et al., 2013). Evidently, septal cells in PRRSV-induced pneumonia were able to produce pro-inflammatory cytokines, IL-1, IL-6, and TNF-α, leading to increase severity of lung pathology (Gomez-Laguna et al., 2010).

2.3.1.2 Humoral immune responses of PRRSV

Following PRRSV infection, PRRSV-specific IgM antibodies were first detected within 5-7 days post infection and declined until undetectable levels at 14-21 days post infection (Park et al., 1995; Yoon et al., 1995; Loemba et al., 1996; Joo et al., 1997). Later, PRRSV-specific IgG antibodies was detected within 7-10 days post infection (Yoon et al., 1995; Loemba et al., 1996) and maintained up to 300 days post infection (Nelson et al., 1994). Unfortunately, the presence of PRRSV-specific antibodies during an early phase of infection does not correlate with protection but

also enhance virus entry into target cells, referred as “antibody-dependent enhancement (ADE)” (Yoon et al., 1996). While, neutralizing antibodies are believed to be the key mechanism for PRRSV protection and/or clearance, they do not appear until approximately one month following PRRSV infection (Loemba et al., 1996; Meier et al., 2003; Lopez and Osorio, 2004). As PRRSV rapidly evolved, thus effective protection requires broad-reactive neutralizing antibodies, or cross neutralizing antibodies (Robinson et al., 2015). Several reports indicated that the ectodomain of PRRSV glycoprotein 5 (GP5) is the major neutralizing epitope (Pirzadeh and Dea, 1997; Ostrowski et al., 2002; Plagemann et al., 2002; Plagemann, 2004). Interestingly, induction of cross neutralization was associated with five candidate sites in GP5, of which three of these sites were located in the first 60 amino acids of the GP5 (Kim et al., 2013). It should be noted that the presence of NAb could not guarantee effective protection. Moreover, the precise NAb titer for sterilizing immunity is still controversial. Nevertheless, NAb plays an important role in reduction of viremia (Osorio et al., 2002) and also correlates well with PRRSV clearance from the lung (Labarque et al., 2000).

2.3.2 Modulation immune response by PRRSV

PRRSV has been recognized as an immunosuppressive virus that exploits various mechanisms to evade the host defense mechanisms. PRRSV infection usually induces delayed innate and adaptive immune responses contributing to persistent infection in the PRRSV infected pigs (Mateu and Diaz, 2008). Previous reports

demonstrated that multiple viral proteins, including NSP1, NSP2, NSP4, NSP11 and N-protein, can suppress type I interferon responses by inhibiting IFN-mediated signaling pathways and blocking the IFN-induced genes activities (Yoo et al., 2010; Huang et al., 2015). Besides, PRRSV can downregulate pro-inflammatory cytokines in the infected pigs (Van Reeth et al., 1999; Van Reeth and Nauwynck, 2000). Interestingly, it has been demonstrated that PRRSV up-regulated IL-10 expression (Suradhat and Thanawongnuwech, 2003) and the upregulation of IL-10 in PRRSV-infected PAMs were found within 12 hour post infection (Genini et al., 2008). In general, IL-10 has been recognized as a potent immunosuppressive cytokine that inhibits pro-inflammatory cytokines and impairs T-cell activation (Moore et al., 2001; Flores-Mendoza et al., 2008). In addition, induction of regulatory T cell (Treg) is one of the most unique mechanisms for modulating swine immune system by PRRSV (Silva-Campa et al., 2009; Wongyanin et al., 2010). Treg plays an important role in maintaining host homeostasis, controlling inflammatory activities and limiting immunopathology (Vignali et al., 2008; Sakaguchi et al., 2009). However, the exaggerated Treg activity can suppress virus-specific T cell responses contributing to ineffective antiviral immune responses and facilitate viral persistence (Zhou, 2008). PRRSV infection is able to establish persistent infection and the virus can persist in various organs, including tonsil, lymph node and lung (Wills et al., 2003). The persistent mechanism contributed to PRRSV persistent is still unclear. It is possible that PRRSV can induce local immunoinhibitory mechanisms which benefit viral persistent in various organs. Notably, in a mouse model, suppression of effective

T-cell response in the lungs and lymph nodes by tissue-resident Treg was reported following respiratory viral infection (Fulton et al., 2010) and pulmonary cryptococcal infection (Wiesner et al., 2016). It is possible that PRRSV infection induce PRRSV-specific Treg at the infected sites, leading to ineffective viral clearance and prolonged infection. Interestingly, it has been shown that PRRSV N-protein is involved in induction of negative immunomodulatory effects. Wongyanin and colleagues demonstrated that PRRSV N-protein induced IL-10 and Treg (Wongyanin et al., 2012).

Evasion mechanisms on neutralizing antibodies have been reported through induction of N-linked glycosylation in the GP3 (Vu et al., 2011) and GP5 proteins (Ansari et al., 2006; Vu et al., 2011). Moreover, induction of decoy GP5 epitope mimicking the major neutralizing epitope resulted in delayed induction of neutralizing antibodies have been reported (Ostrowski et al., 2002). In addition, cell to cell transmission via intercellular nanotubes is an alternative pathway for PRRSV to avoid neutralizing antibodies within the extracellular space (Guo et al., 2016).

2.4 Current problems of PRRSV vaccines

To control PRRSV infection, modified live PRRSV vaccines (MLVs) and killed PRRSV vaccines (KVs) have been approved and are commercially available. MLVs have been widely used for routine vaccination and shown some acceptable outcomes on reducing clinical signs, viremia and virus shedding (Huang et al., 2015). In contrast, KV is safer but provide inefficient protection against either homologous or heterologous

PRRSV strains (Charerntantanakul, 2012; Renukaradhya et al., 2015). HP-PRRSV–based vaccines provided complete protection against the HP-PRRSV challenge in china (Leng et al., 2012; Yu et al., 2015). However, those vaccines were not licensed in other countries. For these reasons, commercially available type 2 MLVs were tested against the HP-PRRSV challenge. However, those vaccines did not completely prevent and control HP-PRRSV infection (Wei et al., 2013; Lager et al., 2014; Do et al., 2015; Charoenchanikran et al., 2016). In addition, the commercial MLV vaccines are able to induce IL-10 production (Diaz et al., 2006; Zuckermann et al., 2007; Park et al., 2014), and Tregs (LeRoith et al., 2011) similar to the natural infection. Thus, elimination of the negative immunomodulatory effects of the PRRSV-MLV should be explored in the future development of an effective PRRSV vaccine (Thanawongnuwech and Suradhat, 2010).

To improve the PRRSV vaccine efficacy, several novel PRRSV vaccines, including a recombinant vaccines, a DNA vaccines, killed and modified live vaccines have been explored. However, those vaccines are not fully protective (Charerntantanakul, 2012). Recently, a novel DNA vaccine has been developed with well-established safety. The vaccine was able to reduce PRRSV-negative immunomodulatory effects (PRRSV-specific Tregs and IL-10) as well as enhance PRRSV-specific IFN-γ responses. However, this vaccine does not contain PRRSV protective epitope (Suradhat et al., 2015; Suradhat et al., 2016). To improve vaccine immunogenicity, the prime-boost immunization regimen

has been utilized to enhance cellular immune responses in animal models, which were reviews elsewhere (Woodland, 2004). Based on this concept, it is of interest to explore if the heterologous DNA-MLV prime-boost immunization would be able to improve the efficacy of the PRRSV-MLV vaccine against the HP-PRRSV strain.

CHAPTER 3

MATERIALS AND METHODS

3.1 Viruses and cells

The Thai isolates, type 2 HP-PRRSV (strain 10PL1) (Ayudhya et al., 2012) and type 2 PRRSV strain (strain 01NP1) (Thanawongnuwech et al., 2004) were provided by the Veterinary Diagnostic Laboratory (CU-VDL), Chulalongkorn University. The viruses were propagated titrated in MARC-145 cells (Thanawongnuwech et al., 1998), and stored at -80 °C until needed.

3.2 Plasmids

The plasmid encoding for truncated PRRSV N protein (pORF7t) was used in the study. Details of cloning procedure, *in vitro* characterization, and plasmid amplification were described in the earlier report (Wongyanin et al., 2012). The immunomodulatory properties of the plasmid have already been reported elsewhere (Suradhat et al., 2015; Suradhat et al., 2016).

3.3 Antibodies and secondary conjugates for flow cytometry

Treg staining system: Anti-swine CD25 mAb (K231.3B2, IgG1) and goat anti-mouse IgG1-FITC were purchased from AbD Serotec (Kidlington, UK). Biotinylated anti-swine CD4 mAb (74-12-4, IgG2b) was purchased from Southern Biotech (Birmingham, AL, USA). Streptavidin-PE was purchased from BioLegend (San Diego, CA, USA). Anti-

human Foxp3-APC conjugate (236A/E7, IgG1) was purchased from eBioscience (San Diego, CA, USA).

IFN-γ and IL-10 staining system: Anti-swine CD3-FITC mAb (BB23-8E6, IgG2b) conjugate was purchased from Southern Biotech. Anti-swine IFN-γ mAb (P2C11) was purchased from BD Biosciences. Anti-swine IL-10 mAb (945A4C437B1, IgG1) was purchased from Biosource (Camarillo, CA, USA). Streptavidin-PETR, goat anti-mouse IgG1-Alexaflur 647 and IgG1 isotype control was purchased from Invitrogen (Carlsbad, CA, USA).

3.4 Animal experiment

Two-week-old, PRRSV-seronegative, crossbred pigs (5-8 pigs/group) were randdomly into 5 groups and identified by ear-tagging. Pigs were immunized transdermally, using Dermavac needleless applicator (kindly provide by Merial Co. Ltd., USA) with DNA vaccine (pORF7t) on day (D) -14 (D -14), followed by intramuscular immunization with a commercial modified live PRRS vaccine (FosteraTM PRRS, Zoetis) at D0. The other experimental groups included pigs received only PBS, trandermally, on D -14 followed by MLV on D0 (MLV), pORF7t on D -14 (DNA), PBS on D0 (PBS) and the negative control groups. At D42 (9 weeks old), all groups, except the negative control group, were intranasally inoculated with 2 ml of 10^4 $TCID_{50}$/ml of HP-PRRSV (strain 10PL1), (0 days post-infection, dpi). Whole blood and serum samples were collected for hematological, serological and virological studies. For immunological

studies, whole blood samples were collected from 5 pigs/group at the indicated time points. All pigs were euthanized and necropsied at 17 dpi, or when found dead or at moribund stage. The pigs were kept in the isolation unit at the Faculty of Veterinary Medicine, Kasetsart University, Kamphangsan campus throughout the experiment. The animal use protocols were conducted under the approval of Chulalongkorn University Animal Care and Use Committee, Chulalongkorn University (Animal Use Protocol No. 1431086).

3.5 Clinical observation, clinical respiratory scores, and pathological examination

Animals were monitored for physical conditions, rectal temperatures and clinical respiratory disease daily during 10 days post infection. Respiratory clinical scores were graded based on the severity of respiratory signs as; 0) normal, 1) mild respiratory distress, 2) moderate respiratory distress, 3) severe respiratory distress with cyanosis. The gross lung lesions and histopathological changes were scored using the previously described protocol (Halbur et al., 1995). Briefly, the microscopic histopathological changes of the lungs were examined and scored as; 0) normal, 1) mild interstitial pneumonia, 2) moderate multifocal interstitial pneumonia, 3) moderate diffuse interstitial pneumonia and 4) severe diffuse interstitial pneumonia.

3.6 Serological assays

Anti-PRRSV antibodies were determined using the commercial ELISA test kit (IDEXX PRRS X3 Ab test, IDEXX, USA) according to the manufacturer's instruction.

Serum samples were considered positive for PRRSV antibodies when the S/P ratio was greater than 0.4.

Serum neutralization (SN) test against the challenged virus was performed using the previously reported protocol (Galliher-Beckley et al., 2015), with minor modification, using 100 $TCID_{50}$ of the type 2 HP-PRRSV (10PL1) as the tested virus. The serum neutralizing antibody (NA) titer of ≥1:2 (1 log_2) was considered as positive. The presence of PRRSV was confirmed by IPMA assay as previously described (Thanawongnuwech et al., 1998).

3.7 Quantification of PRRSV RNA in the sera and lung tissues

Viral RNA was extracted from serum and lung samples using RNA extraction kit (NucleoSpin® RNA virus kit, MACHEREY-NAGEL, Germany). Quantification of PRRSV RNA in the sera was performed using TaqMan® probe-based, real-time RT-PCR as previously described (Egli et al., 2001). RNA Amplification was carried out in a 25 μl reaction containing SuperScript™ III One-Step RT-PCR kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA); 1x Reaction Mix, 0.4 mM of each primer and probe, 0.5 μl of SuperScript® III RT/Platinum Taq Mix and 0.5 μl of viral RNA.

3.8 Isolation of peripheral blood mononuclear cells (PBMC) and flow cytometry

The protocols for porcine PBMC isolation and cellular permeabilization, were performed as previously described (Suradhat et al., 2015). *In vitro* activation was performed in a 96-well plate. Briefly, 1 x 10^6 cells of the isolated PBMC (200 μl) were

plated into each well of a 96-well flat-bottomed plate and incubated with 0.1 multiplicity of infection (m.o.i.) of the type 2 PRRSV (strain 01NP1) or cultured alone or with mock-infected MARC-145 cell lysate for 48 h prior to cell harvesting for fluorescent staining. The staining procedures for Treg, IL-10 and IFN-γ were performed using the previously described protocol (Suradhat et al., 2016). All flow cytometric analyses were performed using the FC 500 MPL System (Beckman Coulter, CA, USA).

3.9 Quantitative polymerase chain reaction (qPCR) of target gene expression in the leukocytes

Total RNA was extracted from peripheral blood mononuclear cells (PBMCs) using the commercial RNA extraction kit (Biotechrabbit, Germany) according to the manufacturer's instructions. The extracted RNA was converted to cDNA with the cDNA synthesis kit (Invitrogen, USA). The levels of cytokine gene expression were determined using SYBR green-based, real-time quantitative PCR. The details of primer sequences were showed in Table 1. The qPCR reaction was performed as previously described (Wongyanin et al., 2010).

Table 1 Oligonucleotide sequences designed for quantitative real-time PCR.

Gene	Oligonucleotide sequences (5'-3')	Product size (bp)
IFNA	F: CTG-GAG-GAG-GAC-TCC-AT	268
	R: GAG-TCT-GTC-TTG-CAG-GTT	
IFNB	F: CAC-CAC-AGC-TCT-TTC-CAT	225
	R: CTG-CAT-CTT-CCT-TCT-GGA-AT	
IL-1β	F: AAC-GTG-CAA-TGA-TGA-CTT-TG	292
	R: CAC-TTC-TCT-CTT-CAA-GTC-CC	
IL-6	F: AGA-ACT-CAT-TAA-GTA-CAT-CCT-CG	180
	R: AGA-TTG-GAA-GCA-TCC-GTC	
GAPDH	F: AAG-TGG-ACA-TTG-TCG-CCA-TC	123
	R: TCA-CAA-ACA-TGG-GGG-CAT-C	

3.10 Statistical analyses

Data was analyzed using analysis of variance (ANOVA) followed by Tukey's multiple comparison tests or Kruskal-Wallis followed by Dunn's multiple comparison tests. All statistical analyses were performed using GraphPad Prism for Windows (GraphPad Software Incorporated, San Diego, CA, USA).

CHAPTER 4

RESULTS

4.1 Clinical evaluation

To investigate the effect of the DNA-MLV prime-boost immunization on clinical protection against the Thai-isolated HP-PRRSV, pigs immunized with indicated treatment were challenged with the Thai-isolated HP-PRRSV on D42. Following HP-PRRSV inoculation, all challenged pigs exhibited clinical signs of HP-PRRSV infection including severe depression, anorexia, lethargy, increased oculonasal discharges, coughing with high fever (Fig. 3 and Fig. 4) starting from 1 dpi. The groups, receiving MLV and DNA-MLV, exhibited delayed clinical signs and lower respiratory scores than the other challenged groups (Fig. 5). Leukopenia was also observed by 2 dpi in the challenged pigs, especially in the group that did not received MLV (Fig. 6). The negative control group remained clinically normal throughout the observation period, indicating that there are no cross-contamination during the experiment. The groups receiving MLV and DNA-MLV immunization exhibited faster recovery, as shown by reduction of body temperature and clinical respiratory scores, than the other challenged groups.

A

B

Figure 3 Clinical presentation of HP-PRRSV infected pigs. Pigs were immunized with the indicated treatment and challenged with HP-PRRSV at 0 dpi. (A) HP-PRRSV infected pigs exhibited clinical symptoms, including severe depression, anorexia with high fever. (B) HP-PRRSV infected pigs also showed swelling of the eyelids with ocular discharge and red discoloration of the ear pinnas.

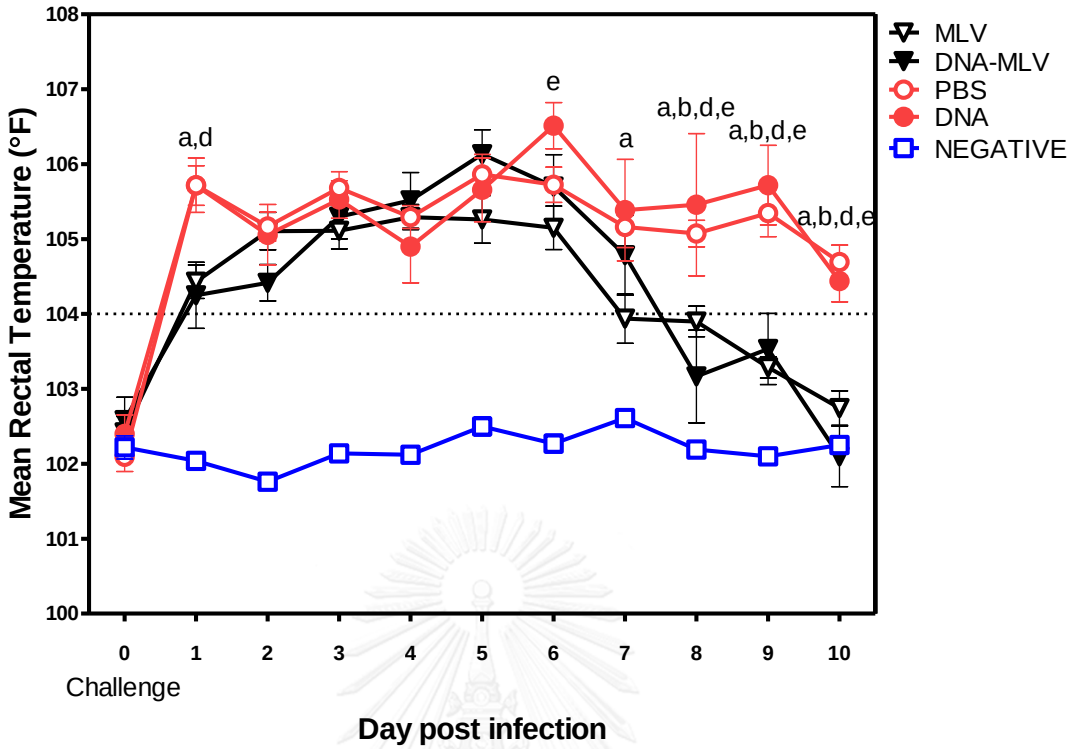


Figure 4 Mean rectal temperatures from the experimental pigs following the HP-PRRSV challenge. Pigs were immunized with the indicated treatment and challenged with HP-PRRSV at 0 dpi. Statistical analyses were performed using one-way ANOVA followed by Tukey's multiple comparison test. [a] indicates difference ($p < 0.05$) between the group receiving MLV and PBS; [b] indicates difference ($p < 0.05$) between the group receiving DNA-MLV and DNA; [c] indicates difference ($p < 0.05$) between the group receiving DNA-MLV and MLV; [d] indicates difference ($p < 0.05$) between the group receiving DNA-MLV and PBS; [e] indicates difference ($p < 0.05$) between the group receiving DNA and MLV; [f] indicates difference ($p < 0.05$) between the group receiving DNA and PBS.

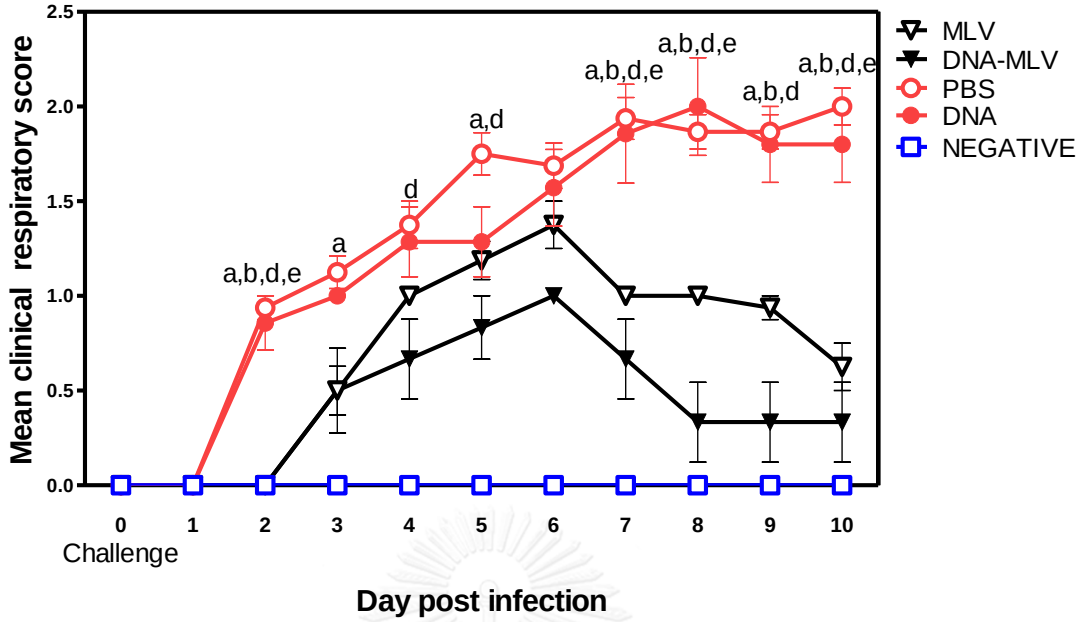


Figure 5 Clinical respiratory scores from the experimental pigs following HP-PRRSV challenge. Pigs were immunized with the indicated treatment and challenged with HP-PRRSV at 0 dpi. Statistical analyses were performed using one-way ANOVA followed by Tukey's multiple comparison test. [a] indicates difference ($p < 0.05$) between the group receiving MLV and PBS; [b] indicates difference ($p < 0.05$) between the group receiving DNA-MLV and DNA; [c] indicates difference ($p < 0.05$) between the group receiving DNA-MLV and MLV; [d] indicates difference ($p < 0.05$) between the group receiving DNA-MLV and PBS; [e] indicates difference ($p < 0.05$) between the group receiving DNA and MLV; [f] indicates difference ($p < 0.05$) between the group receiving DNA and PBS.

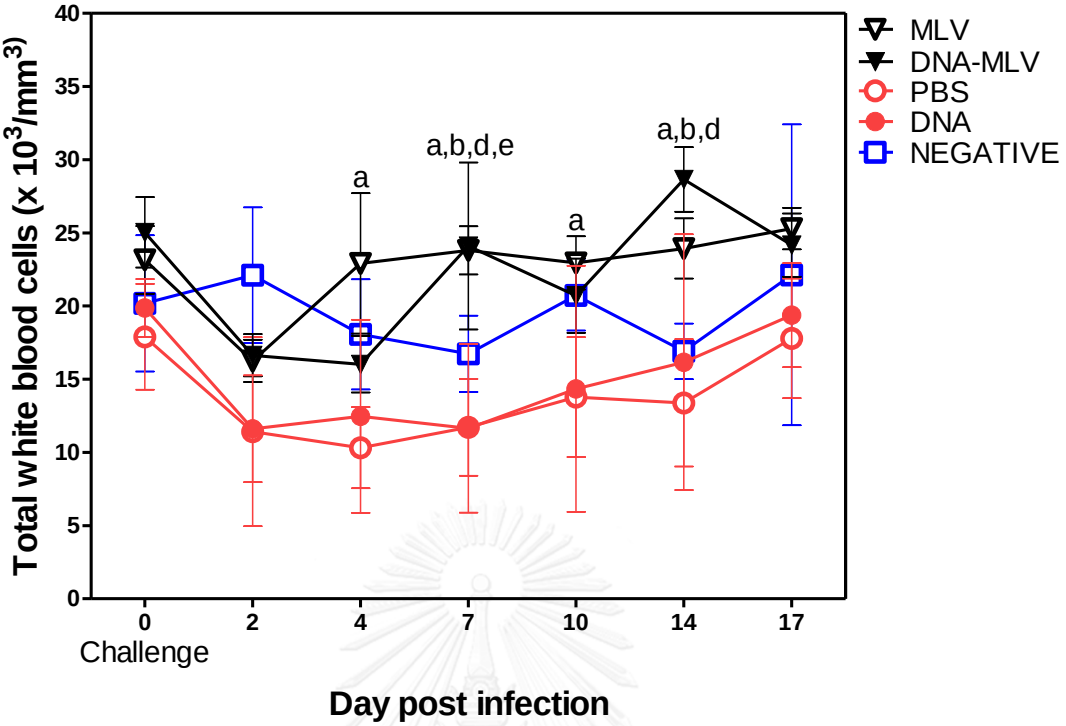


Figure 6 Total white blood cells from the experimental pigs following the HP-PRRSV challenge. Pigs were immunized with the indicated treatment and challenged with HP-PRRSV at 0 dpi. Statistical analyses were performed using one-way ANOVA followed by Tukey's multiple comparison test. [a] indicates difference ($p < 0.05$) between the group receiving MLV and PBS; [b] indicates difference ($p < 0.05$) between the group receiving DNA-MLV and DNA; [c] indicates difference ($p < 0.05$) between the group receiving DNA-MLV and MLV; [d] indicates difference ($p < 0.05$) between the group receiving DNA-MLV and PBS; [e] indicates difference ($p < 0.05$) between the group receiving DNA and MLV; [f] indicates difference ($p < 0.05$) between the group receiving DNA and PBS.

4.2 Virological evaluation

Virological findings were shown in Fig. 4. Following MLV vaccination, the pigs immunized with MLV exhibited viremia during D3-D28.

Following the HP-PRRSV challenge, viremia was detected in all challenged groups from 2 dpi and peaked at 4-7 dpi. The groups that were not immunized with MLV were more viremia than the MLV-immunized groups (DNA-MLV and MLV). Faster reduction of viremia was observed in the groups immunized with MLV and DNA-MLV, while the other challenged groups exhibited prolonged and higher levels of viremia (Fig. 7A). The timing on reduced viral load corresponded well with the reduced body temperatures and clinical signs (Fig. 4 and Fig. 5).

At necropsy, the levels of viral load in the lungs of the MLV and DNA-MLV immunized groups were lower than the other challenged groups (Fig. 7B). There were no differences in the levels of viral load in the sera and lungs between the MLV and DNA-MLV groups (Fig. 7). Immunization with DNA vaccine only did not provided clinical nor virological protection, compared to the non-vaccinated challenged group.

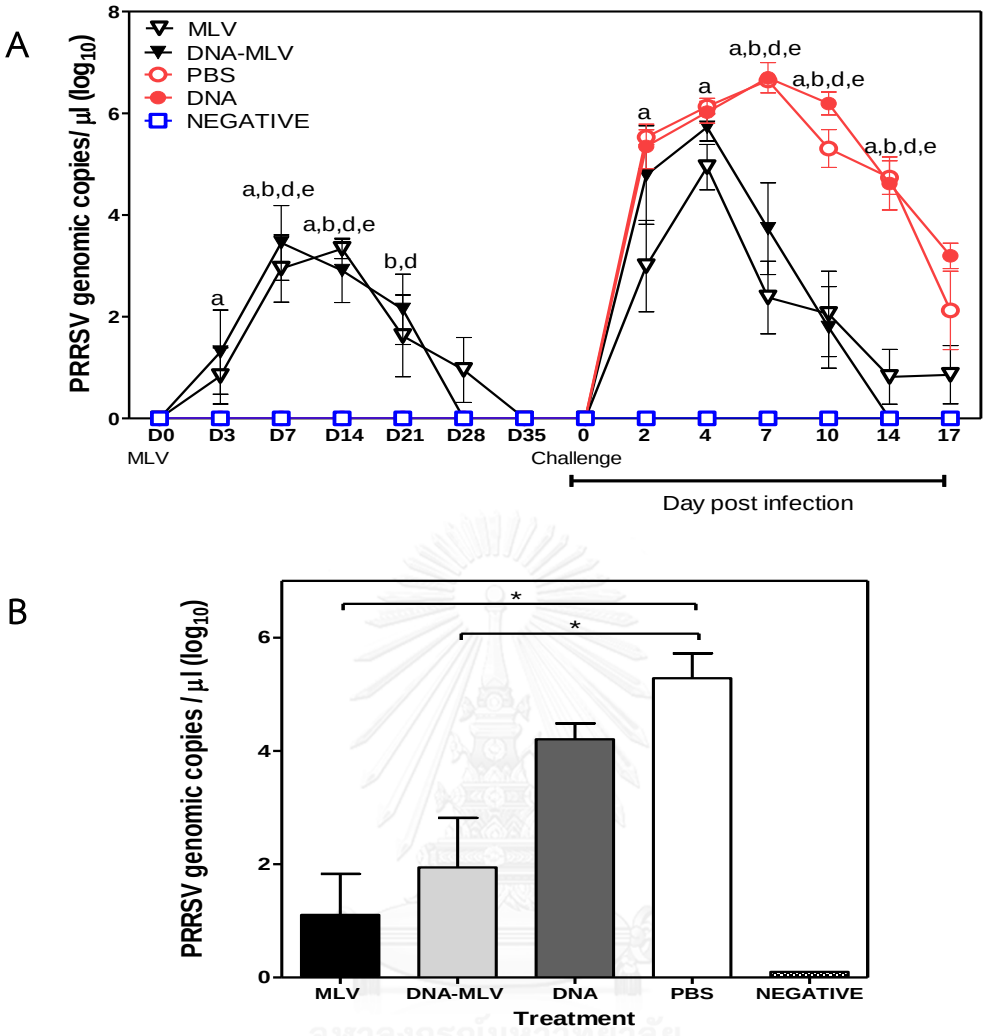


Figure 7 PRRSV genomic copies in the sera (A), and lungs (B) of experimental pigs. Pigs were immunized with the indicated treatment and challenged with HP-PRRSV at 0 dpi. Lung samples were collected at 17 dpi. Statistical analyses were performed using one-way ANOVA followed by Tukey's multiple comparison test. [a] indicates difference ($p < 0.05$) between the group receiving MLV and PBS; [b] indicates difference ($p < 0.05$) between the group receiving DNA-MLV and DNA; [c] indicates difference ($p < 0.05$) between the group receiving DNA-MLV and MLV; [d] indicates difference ($p < 0.05$) between the group receiving DNA-MLV and PBS; [e] indicates difference ($p < 0.05$) between the group receiving DNA and MLV; [f] indicates difference ($p < 0.05$) between the group receiving DNA and PBS.

4.3 Immunomodulatory effects of the heterologous DNA-MLV prime-boost immunization on PRRSV-specific humoral immune responses

The immunomodulatory effects of heterologous DNA-MLV prime-boost immunization on the PRRSV-specific humoral immune responses were demonstrated in Figs. 8-9. Priming of DNA vaccine prior to MLV immunization resulted in enhanced PRRSV-specific antibody responses. Seroconversion, i.e. positive ELISA S/P ratio, was observed in the groups receiving MLV immunization by D14. The DNA-MLV immunized group exhibited higher means S/P ratio than the group received MLV only (Fig. 8). Following the HP-PRRSV challenge, seroconversion was observed in all challenged groups. Interestingly, no anamnestic responses were observed in the MLV and DNA-MLV immunized groups.

No HP-PRRSV specific serum neutralizing (SN) antibodies were detected prior to the HP-PRRSV challenge. Following the HP-PRRSV challenge, increases in SN antibodies were observed in the DNA-MLV (6/6) and MLV (3/8) immunized groups. Significant increase in the SN titers (D0 vs D14) was only observed in the groups receiving DNA-MLV (Fig. 9). The results indicated that heterologous DNA-MLV prime-boost immunization resulted in priming of HP-PRRSV specific neutralizing antibodies.

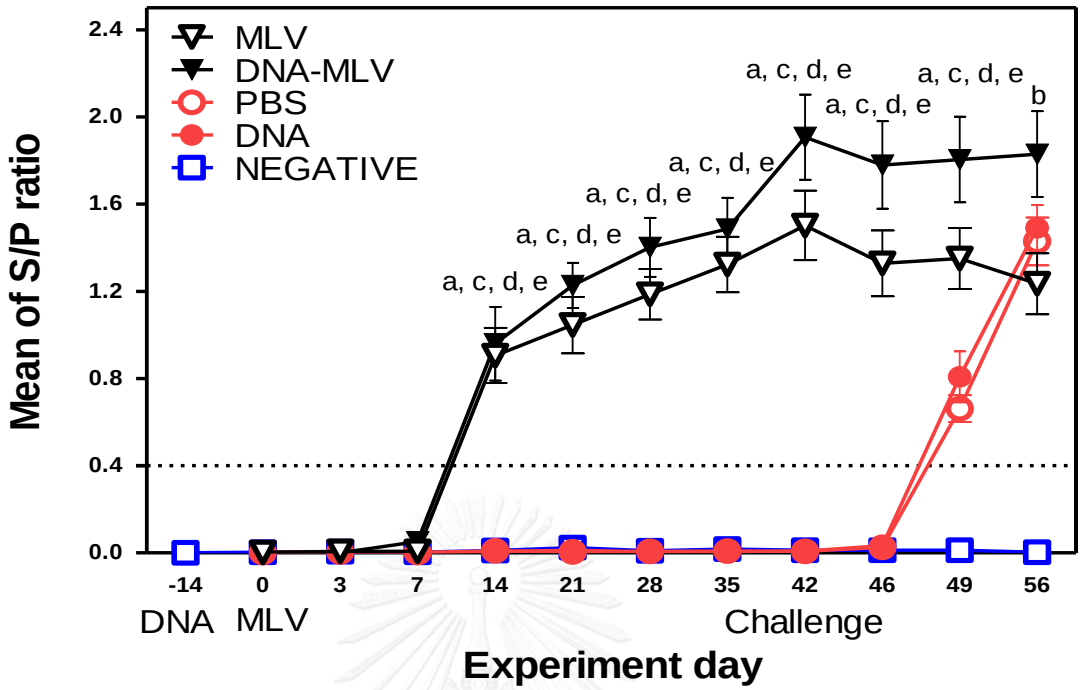


Figure 8 Mean S/P of the experimental pigs. Pigs were immunized with the indicated treatment and challenged with HP-PRRSV at 0 dpi (D42). Statistical analyses were performed using one-way ANOVA followed by Tukey's multiple comparison test. [a] indicates difference ($p < 0.05$) between the group receiving MLV and PBS; [b] indicates difference ($p < 0.05$) between the group receiving DNA-MLV and DNA; [c] indicates difference ($p < 0.05$) between the group receiving DNA-MLV and MLV; d indicates difference ($p < 0.05$) between the group receiving DNA-MLV and PBS; [e] indicates difference ($p < 0.05$) between the group receiving DNA and MLV; [f] indicates difference ($p < 0.05$) between the group receiving DNA and PBS.

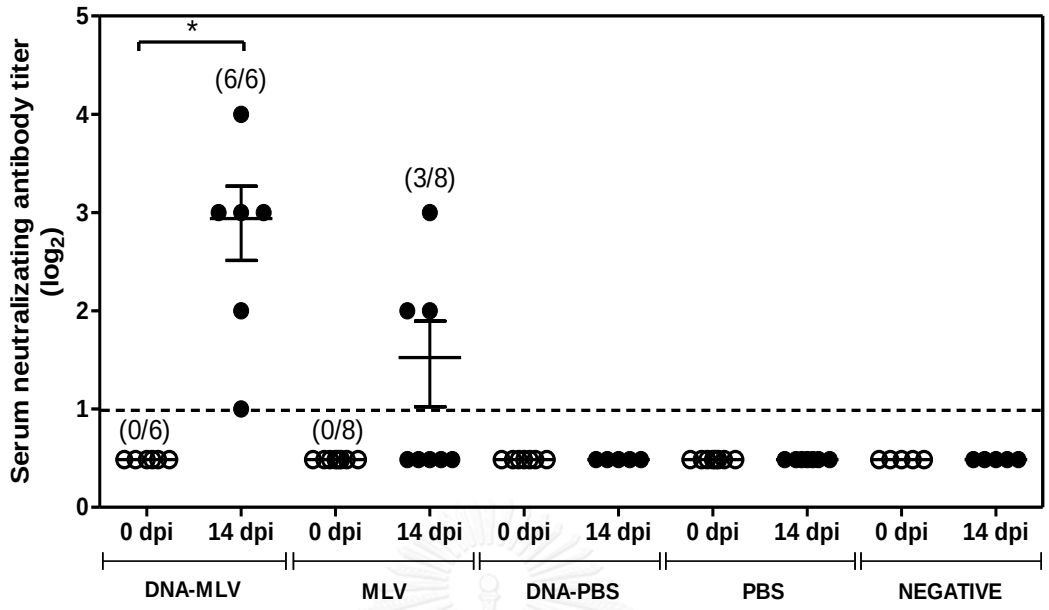


Figure 9 Mean SN antibody titers of the experimental pigs on 0 and 14 dpi. Pigs were immunized with the indicated treatment and challenged with HP-PRRSV at 0 dpi (D42). Statistical analyses were performed using one-way ANOVA followed by Tukey's multiple comparison test. [a] indicates difference ($p < 0.05$) between the group receiving MLV and PBS; [b] indicates difference ($p < 0.05$) between the group receiving DNA-MLV and DNA; [c] indicates difference ($p < 0.05$) between the group receiving DNA-MLV and MLV; d indicates difference ($p < 0.05$) between the group receiving DNA-MLV and PBS; [e] indicates difference ($p < 0.05$) between the group receiving DNA and MLV; [f] indicates difference ($p < 0.05$) between the group receiving DNA and PBS. In Fig. 9, number in parenthesis indicates number of seroconverted pigs/total number in each group. * indicates difference ($p < 0.05$) between the indicated groups.

4.4 immunomodulatory effects of the heterologous DNA-MLV prime-boost immunization the on cellular immune responses

Immunomodulatory effects of heterologous DNA-MLV prime-boost immunization on the cellular immune responses were demonstrated in Fig. 10-16. Following the HP-PRRSV challenge, all challenged groups, especially MLV immunized group, exhibited higher levels of IL-1β gene expression at 2 dpi (Fig. 10) and then gradually declined through the end of observation periods, while enhanced IL-1β gene expression was not observed in the DNA immunized pigs. The levels of *IL-6* gene expression from the PBS group began to rise during the first few days and remained high through the end of observation period. On the other hand, the levels of *IL-6* gene expression from DNA-MLV immunized group were lower than the MLV group at 4 dpi. The levels of *IL-6* gene expression from the DNA group were also lower than the MLV immunized and the PBS groups at 4 dpi and maintained at the lower level (Fig. 11). Interestingly, pigs received prime-boost, DNA-MLV, immunization exhibited significantly higher levels of the anti-viral cytokine, IFN-α gene expression following the challenge, at 2 dpi (Fig. 12), while enhanced IFN-α gene expression was not observed in the MLV immunized pigs. Likewise, the levels of IFN-β gene expression in the DNA-MLV immunized group were up-regulated at 4 dpi, compared to the other challenged groups, and then gradually declined through the end of observation periods (Fig. 13). However, there were no significant differences between the groups. Following MLV

immunization, increased numbers of PRRSV-specific $CD3^+IFN\text{-}\gamma^+$ cells were observed in the DNA-MLV immunized group during D0-D28. Following the HP-PRRSV challenge, the numbers of PRRSV-specific $CD3^+$ $IFN\text{-}\gamma^+$ cells in all challenged groups increased to the comparable levels (Fig. 14).

The heterologous prime-boost DNA-MLV immunization resulted in reduction of negative immunomodulatory effects following MLV vaccination. As shown in Fig. 15 and 16, the numbers of PRRSV-specific IL-10 producing cells and PRRSV-specific Treg in the DNA-MLV immunized group were lower than those receiving MLV immunization. This effect was also observed following the HP-PRRSV challenge. Interestingly, the group that received only DNA vaccine also exhibited better control of Treg induction following the HP-PRRSV challenge. Together, the data indicated that priming with pORF7t provided positive immunomodulatory effects on the cellular immune responses induced by the MLV, resulted in improved PRRSV-specific cellular immune responses following the HP-PRRSV challenge.

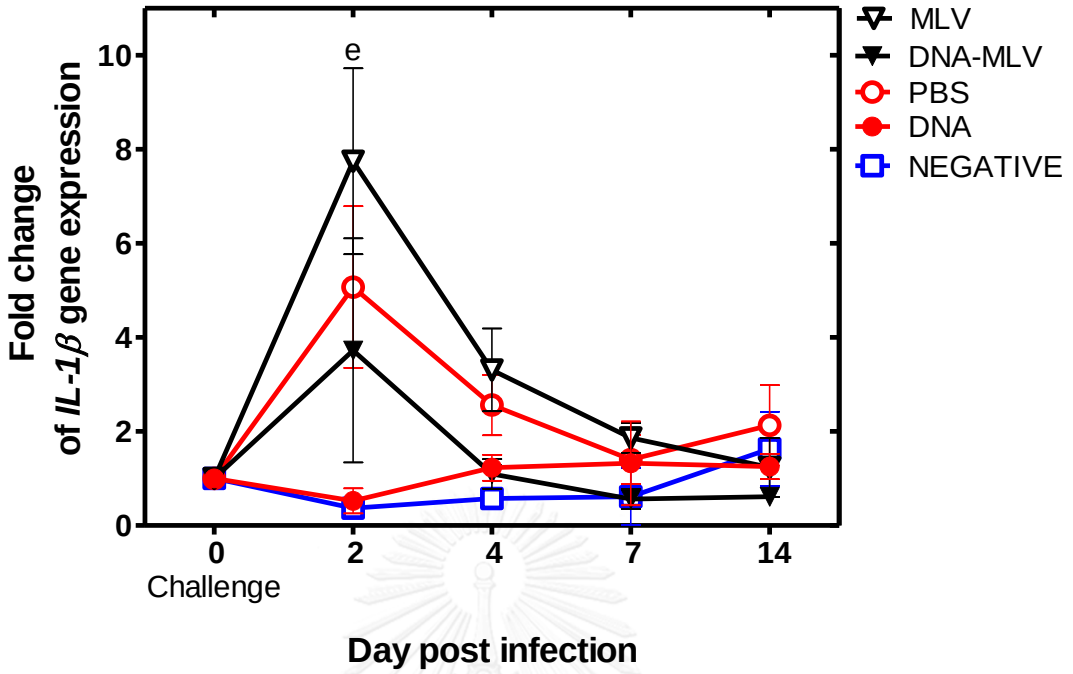


Figure 10 Levels of IL-1β gene expression in the experimental pigs. Pigs were immunized with the indicated treatment and challenged with HP-PRRSV at 0 dpi (D42). Statistical analyses were performed using one-way ANOVA followed by Tukey's multiple comparison test. [a] indicates difference ($p < 0.05$) between the group receiving MLV and PBS; [b] indicates difference ($p < 0.05$) between the group receiving DNA-MLV and DNA; [c] indicates difference ($p < 0.05$) between the group receiving DNA-MLV and MLV; d indicates difference ($p < 0.05$) between the group receiving DNA-MLV and PBS; [e] indicates difference ($p < 0.05$) between the group receiving DNA and MLV; [f] indicates difference ($p < 0.05$) between the group receiving DNA and PBS.

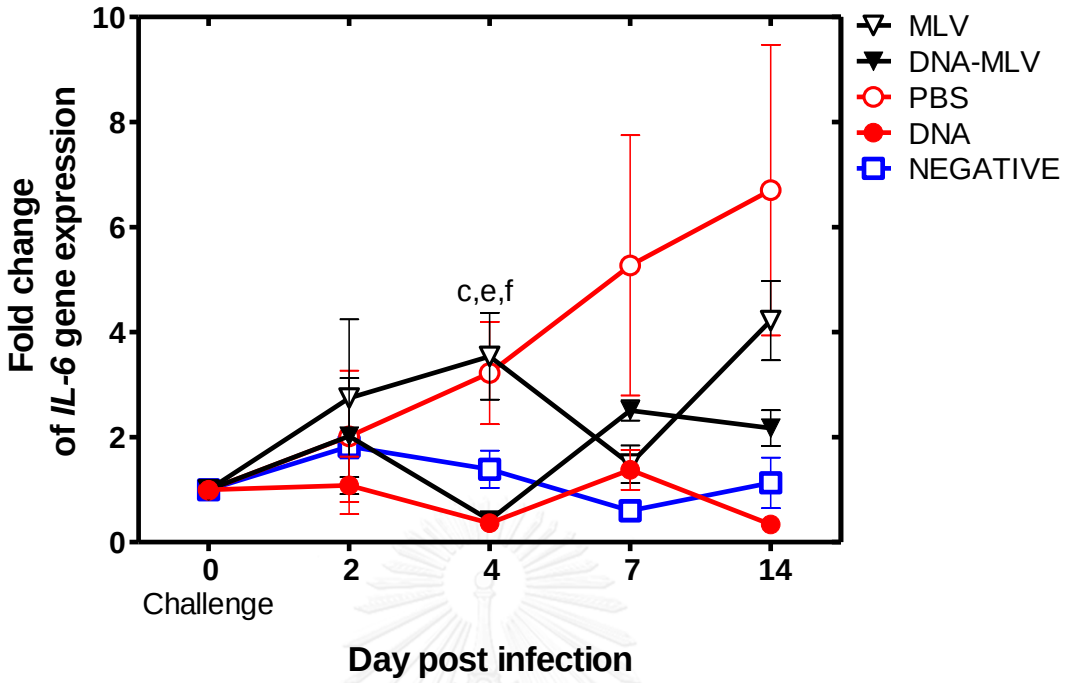


Figure 11 Levels of IL-6 gene expression in the experimental pigs. Pigs were immunized with the indicated treatment and challenged with HP-PRRSV at 0 dpi (D42). Statistical analyses were performed using one-way ANOVA followed by Tukey's multiple comparison test. [a] indicates difference ($p < 0.05$) between the group receiving MLV and PBS; [b] indicates difference ($p < 0.05$) between the group receiving DNA-MLV and DNA; [c] indicates difference ($p < 0.05$) between the group receiving DNA-MLV and MLV; d indicates difference ($p < 0.05$) between the group receiving DNA-MLV and PBS; [e] indicates difference ($p < 0.05$) between the group receiving DNA and MLV; [f] indicates difference ($p < 0.05$) between the group receiving DNA and PBS.

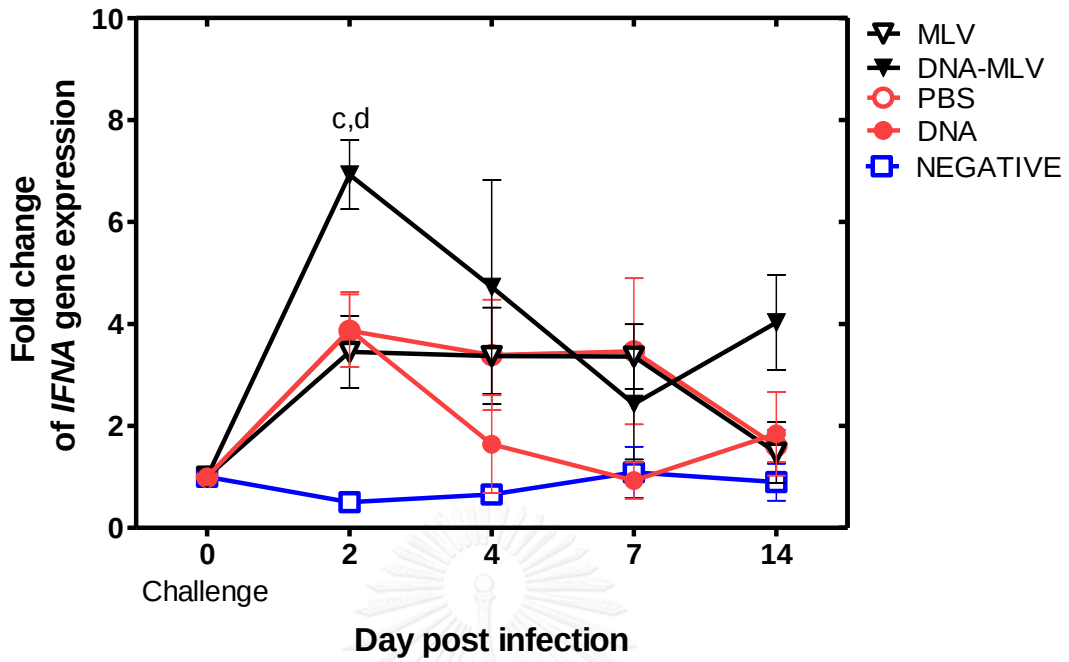


Figure 12 Levels of IFNA gene expression in the experimental pigs. Pigs were immunized with the indicated treatment and challenged with HP-PRRSV at 0 dpi (D42). Statistical analyses were performed using one-way ANOVA followed by Tukey's multiple comparison test. [a] indicates difference ($p < 0.05$) between the group receiving MLV and PBS; [b] indicates difference ($p < 0.05$) between the group receiving DNA-MLV and DNA; [c] indicates difference ($p < 0.05$) between the group receiving DNA-MLV and MLV; d indicates difference ($p < 0.05$) between the group receiving DNA-MLV and PBS; [e] indicates difference ($p < 0.05$) between the group receiving DNA and MLV; [f] indicates difference ($p < 0.05$) between the group receiving DNA and PBS.

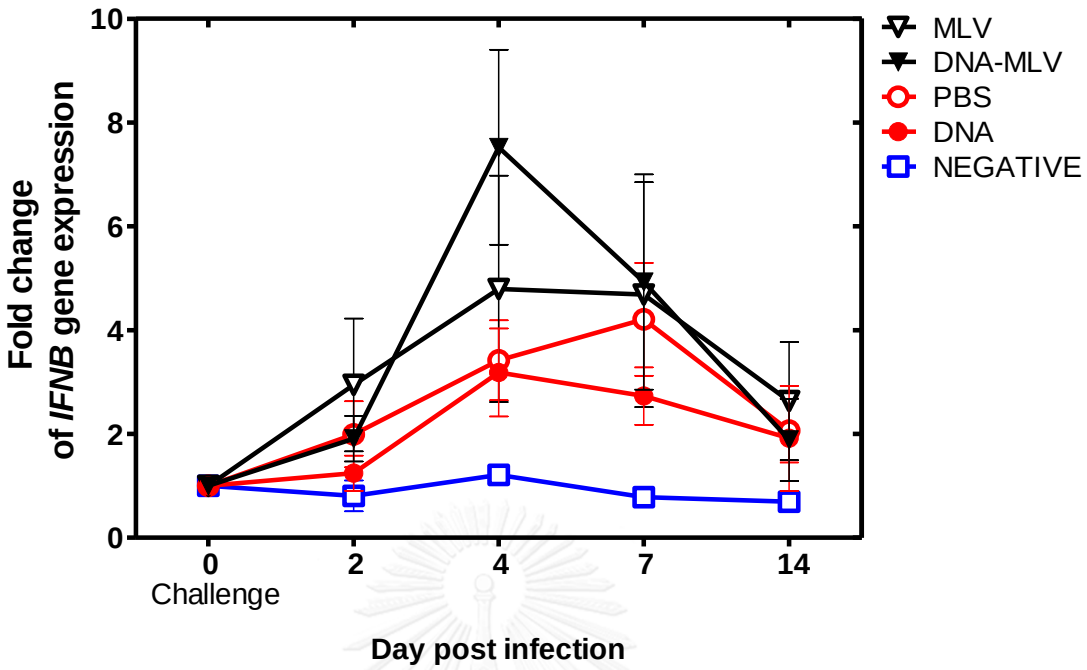


Figure 13 Levels of IFNB gene expression in the experimental pigs. Pigs were immunized with the indicated treatment and challenged with HP-PRRSV at 0 dpi (D42). Statistical analyses were performed using one-way ANOVA followed by Tukey's multiple comparison test. [a] indicates difference ($p < 0.05$) between the group receiving MLV and PBS; [b] indicates difference ($p < 0.05$) between the group receiving DNA-MLV and DNA; [c] indicates difference ($p < 0.05$) between the group receiving DNA-MLV and MLV; d indicates difference ($p < 0.05$) between the group receiving DNA-MLV and PBS; [e] indicates difference ($p < 0.05$) between the group receiving DNA and MLV; [f] indicates difference ($p < 0.05$) between the group receiving DNA and PBS.

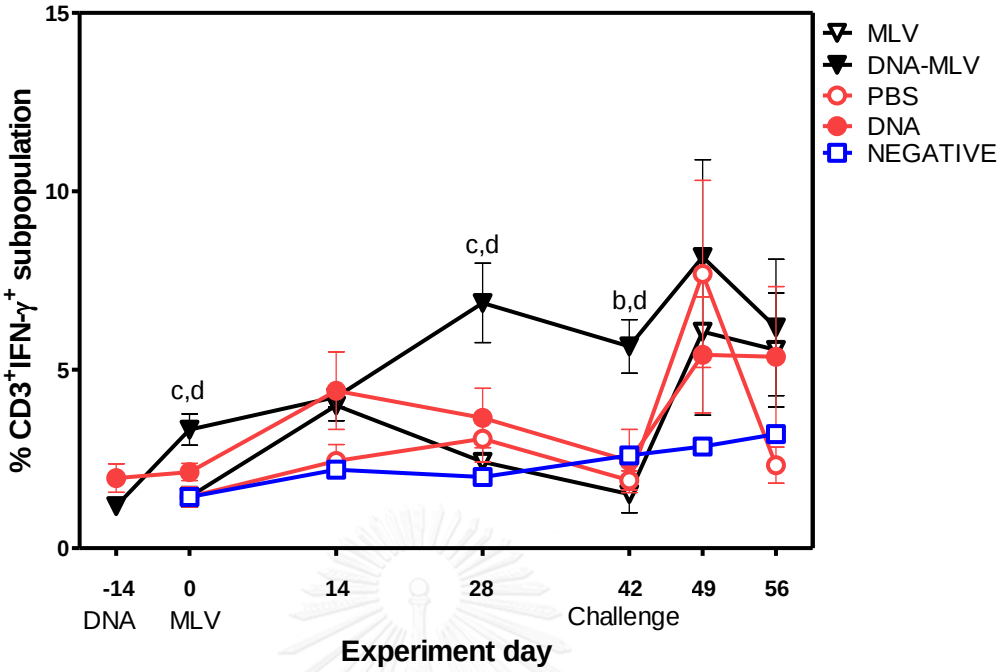


Figure 14 Percentages of PRRSV-specific CD3$^+$IFN-γ$^+$ subpopulation in the PBMC of experimental pigs. Pigs were immunized with the indicated treatment and challenged with HP-PRRSV at 0 dpi (D42). Statistical analyses were performed using one-way ANOVA followed by Tukey's multiple comparison test. [a] indicates difference ($p < 0.05$) between the group receiving MLV and PBS; [b] indicates difference ($p < 0.05$) between the group receiving DNA-MLV and DNA; [c] indicates difference ($p < 0.05$) between the group receiving DNA-MLV and MLV; d indicates difference ($p < 0.05$) between the group receiving DNA-MLV and PBS; [e] indicates difference ($p < 0.05$) between the group receiving DNA and MLV; [f] indicates difference ($p < 0.05$) between the group receiving DNA and PBS.

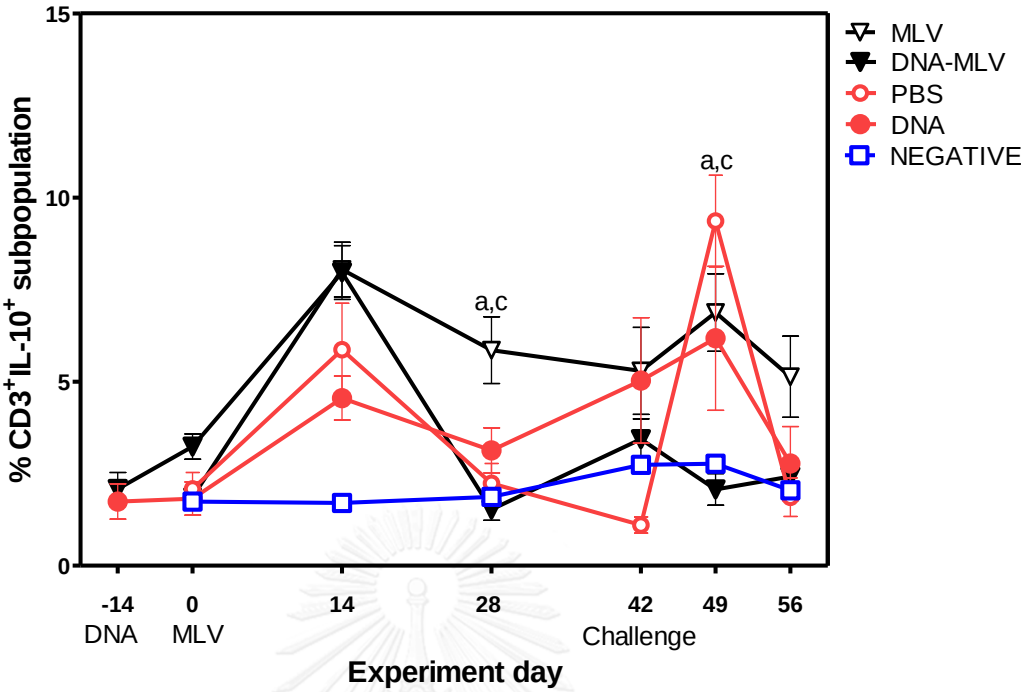


Figure 15 Percentages of PRRSV-specific $CD3^{+}IL\text{-}10^{+}$ subpopulation in the PBMC of experimental pigs. Pigs were immunized with the indicated treatment and challenged with HP-PRRSV at 0 dpi (D42). Statistical analyses were performed using one-way ANOVA followed by Tukey's multiple comparison test. [a] indicates difference ($p < 0.05$) between the group receiving MLV and PBS; [b] indicates difference ($p < 0.05$) between the group receiving DNA-MLV and DNA; [c] indicates difference ($p < 0.05$) between the group receiving DNA-MLV and MLV; d indicates difference ($p < 0.05$) between the group receiving DNA-MLV and PBS; [e] indicates difference ($p < 0.05$) between the group receiving DNA and MLV; [f] indicates difference ($p < 0.05$) between the group receiving DNA and PBS.

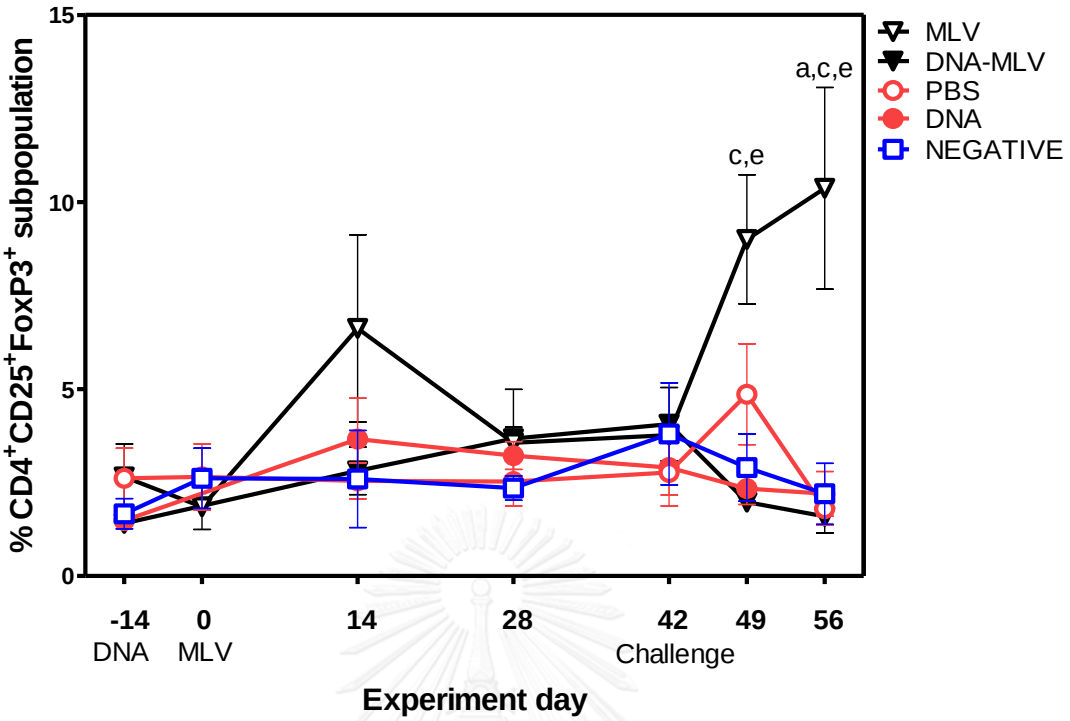


Figure 16 Percentages of PRRSV-specific $CD4^+CD25^+FoxP3^+$ subpopulation in the PBMC of experimental pigs. Pigs were immunized with the indicated treatment and challenged with HP-PRRSV at 0 dpi (D42). Statistical analyses were performed using one-way ANOVA followed by Tukey's multiple comparison test. [a] indicates difference ($p < 0.05$) between the group receiving MLV and PBS; [b] indicates difference ($p < 0.05$) between the group receiving DNA-MLV and DNA; [c] indicates difference ($p < 0.05$) between the group receiving DNA-MLV and MLV; d indicates difference ($p < 0.05$) between the group receiving DNA-MLV and PBS; [e] indicates difference ($p < 0.05$) between the group receiving DNA and MLV; [f] indicates difference ($p < 0.05$) between the group receiving DNA and PBS.

4.5 Pathological evaluation

The numbers of the survived pigs in each group, microscopic and macroscopic pathological scores at 17 dpi or at the time of death were summarized in Table 2. HP-PRRSV-induced pneumonia were characterized by failed to collapse, diffuse consolidation, diffusely tan or mottled-tan consolidation of the lungs with enlargement of tracheobronchial lymph nodes (Fig. 17). At the end of the observation period (17 dpi), the pig immunized with MLV and DNA-MLV exhibited better gross lung lesions than the other challenge groups (Table 2). Upon lung microscopic examination, there were no differences in the mean microscopic lesions among the groups (Table 2). All challenged groups showed mild or moderate degree of interstitial pneumonia that characterized by septal infiltration by lymphocytes and histiocytes and mild peribronchiolar lymphoid hyperplasia (Fig. 18).

Table 2 Numbers of experimental pigs, gross lung lesion scores and microscopic lung lesion scores among the experimental groups.

Treatment	n (0 dpi)	n (17 dpi)	Gross lung lesion score† (mean ± SEM)	Mean microscopic lung lesions† (mean ± SEM)
MLV	8	8	5.50 ± 2.28[a,e]	1.25 ± 0.164
DNA-MLV	6	6	3.20 ± 2.33[b,d]	1.83 ± 0.167
DNA	8	4	40.00 ± 7.70	2.25 ± 0.479
PBS	8	7	31.63 ± 5.80	2.00 ± 0.218
Negative	5	5	0.00	1.40 ± 0.245

† Gross lung lesions score and microscopic lung lesions score were evaluated based on the previously study (Halbur et al., 1995)

Statistical analyses were performed using one-way ANOVA followed by Tukey's multiple comparison test. [a] indicates difference ($p < 0.05$) between the group receiving MLV and PBS; [b] indicates difference ($p < 0.05$) between the group receiving DNA-MLV and DNA; [c] indicates difference ($p < 0.05$) between the group receiving DNA-MLV and MLV; [d] indicates difference ($p < 0.05$) between the group receiving DNA-MLV and PBS; [e] indicates difference ($p < 0.05$) between the group receiving DNA and MLV; [f] indicates difference ($p < 0.05$) between the group receiving DNA and PBS.

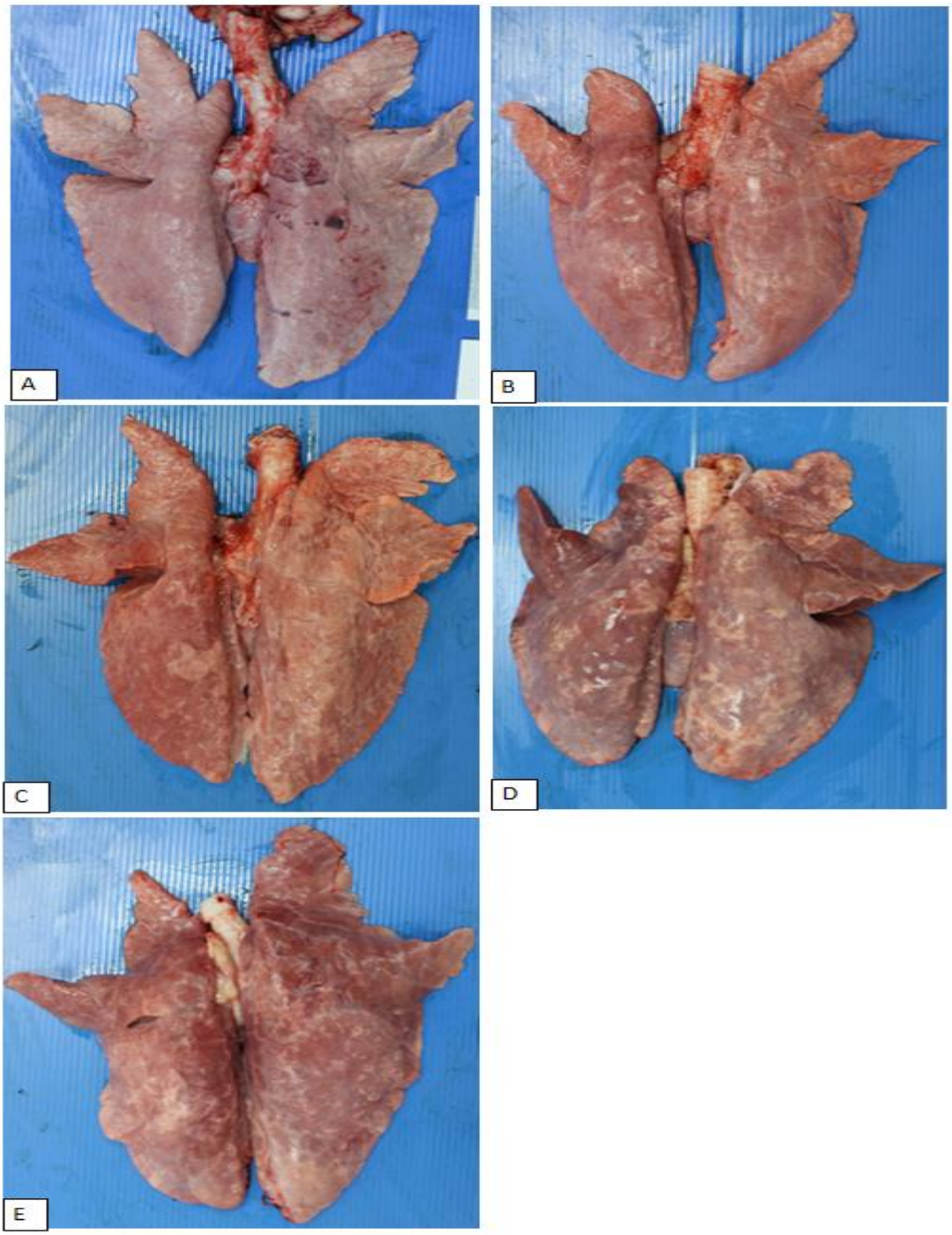


Figure 17 Gross lung lesions of the experimental pigs at 17 dpi Representative of a normal lung from a negative control pig (A), Representative of gross lung lesions from pigs received MLV (B), DNA-MLV (C), PBS (D), and DNA (E).

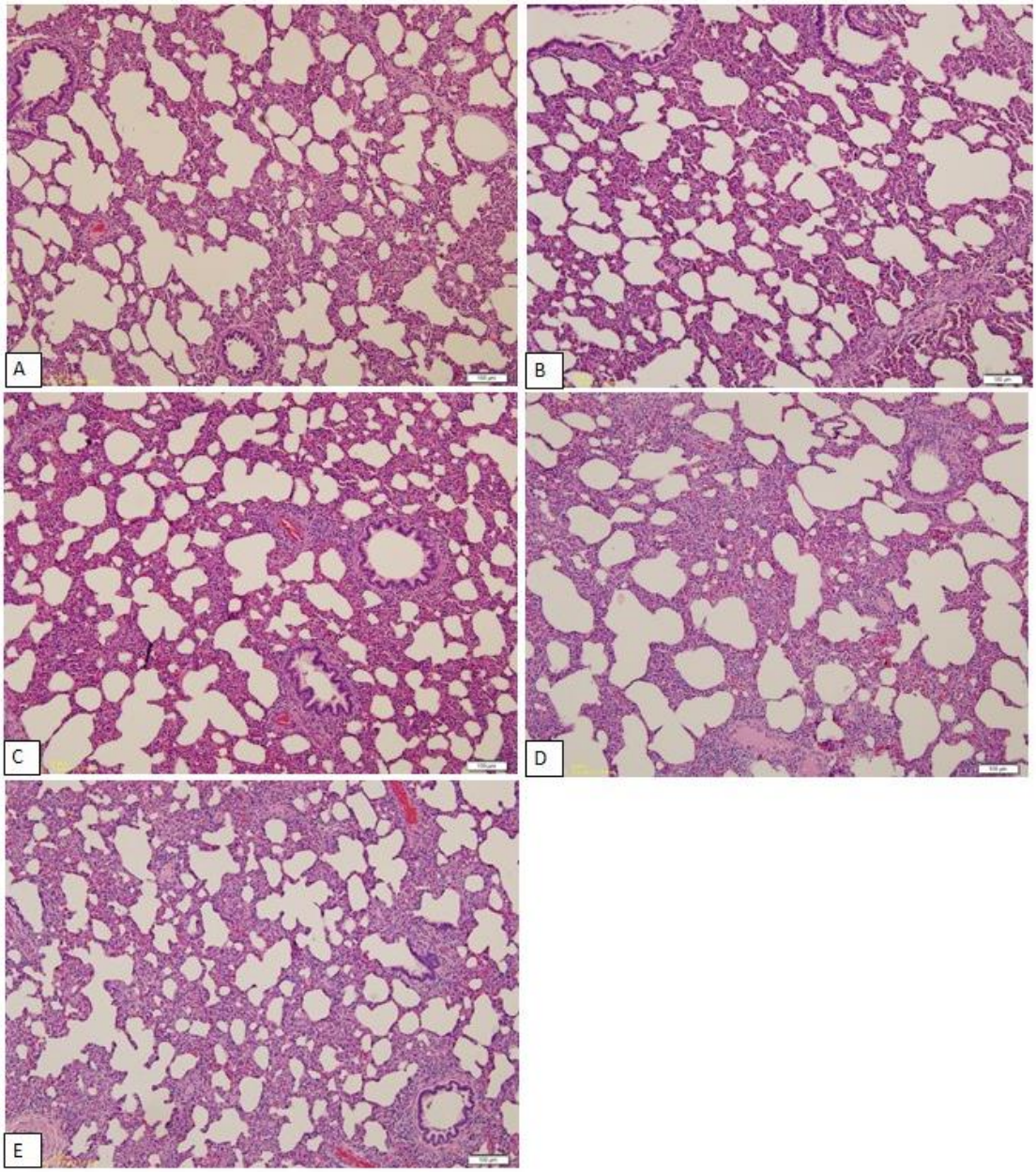


Figure 18 Histopathological findings of the lung tissues from the negative control pig (A), pigs that received MLV (B), DNA-MLV (C), PBS (D), and DNA (E). Interstitial pneumonia is characterized by thickening of alveolar septa by increased infiltration of lymphocytes and histiocytes. The lung tissues were stained with hematoxylin and eosin (H&E).

4.6 General summary

Taken together, the prime-boost, DNA-MLV, immunization regimen could alter the patterns of PRRSV-specific immune responses by enhancing both cellular and humoral immunity, and reducing negative immunomodulatory effects induced by MLV and HP-PRRSV. The DNA-MLV immunization could provide partial clinical protection with faster viral clearances. However, there were no differences in degree of clinical protection between the groups immunized with MLV and DNA-MLV. Thus, priming with DNA vaccine did not provide any additional benefit on clinical protection against the HP-PRRSV infection. The effects of the prime-boost, DNA-MLV, immunization were summarized in Table 3.

Table 3 The immunomodulatory effects of DNA-MLV immunization obtained from this study.

Parameters	DNA-MLV	MLV	DNA
Clinical signs			
Viral load			
ELISA titer			
SN titer			
Type I IFN			
CMI (IFN-γ)			
IL-10			
Treg			

indicate positive effect indicate negative effect or no effect

CHAPTER 5

DISCUSSION AND CONCLUSION

5.1 Discussion

In this study, we hypothesized that the heterologous DNA-MLV prime-boost immunization should enhance the efficacy of the MLV against the HP-PRRSV. Our results demonstrated that the DNA vaccine could improve the quality of PRRSV-specific immunity in the pigs that received DNA-MLV immunization. However, priming with the DNA vaccine did not provide significant advantage on clinical protection over the MLV. As the DNA vaccine was designed to reduce PRRSV-induced negative immunomodulatory effects, the efficacy of the DNA-MLV immunization was still mainly relied on the MLV-induced protective mechanisms. The findings that the current commercially available MLV provided only partial protection against the HP-PRRSV challenges are consistent to the previous reports (Lager et al., 2014; Do et al., 2015; Charoenchanikran et al., 2016). It is indicated that immunization with PRRS-MLV derived from the same (lineage 8) as HP-PRRSV could not guarantee complete protection. It is possible that nature of HP-PRRSV and the intra-lineage genetic/antigenic differences between the vaccine virus (lineage 8) and the challenged HP-PRRSV (lineage 8, sublineage 8.7) involve the vaccine efficacy. From our point of view, proper selection of PRRSV vaccine in relation to a PRRSV field strain together with

strict biosecurity and management, could provide substantial benefits for prevention and control of HP-PRRSV or PRRSV in infected areas.

Interestingly, priming with DNA vaccine also enhanced the anti-viral cytokine, IFN-α, production following HP-PRRSV infection (Fig. 12). The N-protein of PRRSV has been reported as an IFN-antagonist (Huang et al., 2014; Huang et al., 2015; Lunney et al., 2016). It was possible that priming with the DNA vaccine resulted in enhanced immunity to the N protein which resulted in reduced IFN-antagonist activity following the HP-PRRSV infection. Previous reports indicated that PRRSV-specific IFN-γ secreting cells are the main protective mechanism against the PRRSV infection (Meier et al., 2003; Zuckermann et al., 2007; Park et al., 2014). In this study, increased numbers of $CD3^{+}IFN\text{-}\gamma^{+}$ cells in the DNA-MLV group were observed both following MLV immunization and HP-PRRSV challenge, indicating that the broad immunomodulatory effects of the DNA vaccine on induction of the cellular immunity. Our findings indicated that the appearance of HP-PRRSV-specific IFN-γ producing cells coincided with the reduction of viremia in the immunized pigs. These findings supported the previous studies that Induction of HP-PRRSV-specific IFN-γ production may provide partial protection, resulted in reduced severity of clinical signs and viremic levels (Do et al., 2015). Nonetheless, our results indicated that the well-primed cellular immunity was not sufficient to completely protect HP-PRRSV infection.

It should be noted that most of modified live type 2 PRRS vaccines failed to prime for neutralizing antibody against the HP-PRRSV (Do et al., 2015; Galliher-Beckley et al., 2015). In this study, induction of SN antibodies was observed following the challenge and correlated well with clinical recovery and viral clearance in the infected pigs. Priming with the DNA vaccine could significantly enhance neutralizing antibodies in the immunized pigs, suggesting the positive immunomodulatory effect of the DNA vaccine on PRRSV-specific humoral immunity. The priming of SN antibodies observed in the MLV immunized groups, regardless of the DNA priming could be due to the conserved neutralizing epitopes among the PRRSV lineages. The MLV used in this study and the challenged HP-PRRSV are from lineage 8 (Shi et al., 2010; Do et al., 2015). Previous report indicated the existence of cross neutralization epitopes located in the first 60 amino acids of the GP5 proteins among the type 2 PRRSV strains (Kim et al., 2013). It is speculated that both HP-PRRSV and PRRS MLV shared common neutralizing epitopes, leading to priming and induction of cross-neutralizing antibodies following the challenge. The findings also highlighted the significance of SN antibodies on protection against HP-PRRSV. For better clinical protection, the future vaccines against the HP-PRRSV should effectively elicit SN antibodies prior to viral exposure.

The severity of lung lesions induced by HP-PRRSV infection was higher than the low-virulent PRRS strain (Guo et al., 2013; Zhang et al., 2013; Galliher-Beckley et al., 2015). In this study, pneumonia induced HP-PRRSV is consistent to the previous HP-

PRRSV studies (Tian et al., 2007; Guo et al., 2013; Do et al., 2015; Charoenchanikran et al., 2016). The finding on upregulation of the induction of pro-inflammatory cytokine gene expression following HP-PRRSV infection is consistent to the previous report. Several studies indicated that HP-PRRSV infected pigs exhibited earlier and higher levels of pro-inflammatory cytokines, including IL-1, IL-6 and IFN-α (Liu et al., 2010; Zhang et al., 2013). Upregulation of these cytokines contributed to aggravated inflammation and lung pathology (Gomez-Laguna et al., 2010; Liu et al., 2010; Guo et al., 2013). One of the unique clinical presentations following HP-PRRSV infection is high fever (Tian et al., 2007). It is possible that up-regulation of the pyrogenic cytokines, including IL-1 and IL-6, resulted in increased prostaglandin E2 (PGE2) production that caused fever (Saper and Breder, 1994; Dinarello, 2004). In addition, high levels of viremia were associated with severity of interstitial pneumonia (Johnson et al., 2004; Han et al., 2013). In this study, reduction of HP-PRRSV viremia correlated with the reduction of lung lesion scores and lower levels of virus load in the lung tissues at the end of experiment. It should be noted that reduction of HP-PRRSV viremia was achieved by the MLV immunization. The reduction of viremic could be due to the induction of neutralizing antibodies. Our data showed that the levels of viremia significantly decreased on the appearance of viral-specific neutralizing antibodies. This finding is consistent with the previous studies that neutralizing antibodies could prevent viremia and provide protection against PRRSV infection (Yoon et al., 1996; Lopez and Osorio, 2004). In addition, induction of PRRSV-specific IFN-γ secreting cells after HP-PRRSV

challenge reflected enhanced anti-viral cell-mediated immunity for viral clearance (Meier et al., 2003; Loving et al., 2008; Park et al., 2014). Thus, induction of neutralizing antibodies and PRRSV-specific IFN-γ secreting cells observed in this study should resulted in enhanced anti-viral immunity and subsequently reduced viremia and lung lesions in the immunized pigs. Our findings indicated that DNA-MLV immunization could elicit host immune responses and reduce negative immunomodulatory effects of the MLV. However, effective protection against HP-PRRSV infection may require earlier and higher titer of HP-PRRSV-specific neutralizing antibodies. In the future study, PRRSV vaccine development should focus on induction of broad reactive and potent neutralizing antibodies as well as reduction of the negative immunomodulatory effects. The vaccine will be useful for disease prevention and control in PRRSV endemic areas.

5.2 Conclusion

Priming with DNA vaccine (pORF7t) followed by MLV vaccination provided positive immunological outcomes by enhancing HP-PRRSV specific immunity, and reducing negative immunomodulatory effects following MLV vaccination and HP-PRRSV infection. However, there were no differences in degree of clinical protection between the groups immunized with MLV and DNA-MLV. These findings could benefit for vaccine development and establishment of PRRSV control strategies in the future.

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APPENDIX

Appendix A

Preparation of reagents

1. 10X MEM

1.1 MEM powder (for 10 liters)	1 bottle
1.2 Distilled water to	1000 ml

2. MEM complete media

2.1 10X MEM	100 ml
2.2 L-glutamine	10 ml
2.3 Antibiotic (Penicillin Streptomycin)	1 ml
2.4 Pyruvic acid	0.11 g
2.5 $NaHCO_3$	2 g
2.6 Distilled water to	1000 ml

3. RPMI complete media

3.1 Fetal bovine serum (FBS)	50 ml
3.2 100x L-glutamine	5 ml
3.3 100x antibiotic/antimycotic	5 ml
3.4 HEPES	12.5 ml
3.5 50 mM β-mercaptoethanol	0.5 ml
3.6 Advance RPMI-1640 to	500 ml

Appendix B

Protocol for serum neutralization test

1. Heat inactivation of serum at 56 °C for 30 min
2. Prepare a two-fold dilution of serum samples until reach 1/256 dilution
3. Add with equal volume of HP-PRRSV (strain 10PL01) containing 100 $TCID_{50}$ of virus in the serial dilution of serum
4. Incubate at 37 °C for 1 hr in a humidified atmosphere containing 5% CO_2
5. Transfer the mixtures to 96-well plates of MARC-145
6. Incubate for 48-72 hr at 37 °C in a humidified atmosphere containing 5% CO_2
7. Determine end-point titers using immunoperoxidase monolayer assay (IPMA)

APPENDIX C

Protocol for indirect immunoperoxidase monolayer assay (IPMA)

1. Add 50 μl/well of 4% formaldehyde (in PBS) fixation for 30 minutes at room temperature
2. Wash plate three times with 0.5% PBS Tween 20
3. Incubate with primary antibodies, mAb SDOW17 (1:1000) and mAb SR30 (1:1000), in 1% bovine serum albumin (BSA) in PBS by transferring 30 μl/well for 1 hr at room temperature
4. Wash plate three times with 0.5% PBS Tween 20
5. Incubate with secondary antibody, polyclonal rabbit anti-mouse HRP conjugate (1:300), in 1% bovine serum albumin (BSA) in PBS by transferring 30 μl/well for 1 hr at room temperature
6. Wash plate three times with 0.5% PBS Tween 20
7. Incubate with AEC peroxidase substrate solution by transferring 50 μl/well for 30 minutes at room temperature
8. Wash plate three times with tap water and dry plate

VITA

Mr. Chaitawat Sirisereewan was born on January 29, 1989 at Ayutthaya province. He graduated with Doctor of Veterinary Medicine, first class honor, from Faculty of Veterinary Science Chulalongkorn University in 2014. Soon after graduated, He enrolled in the Master program in Veterinary Pathobiology program since 2014.

During the Master's degree study, He received a scholarship from Chulalongkorn University Graduate Scholarship to commemorate the 72nd anniversary of His Majesty King Bhumibol Adulyadej.

Academic publication

Sirisereewan C, Nedumpun T, Kesdangsakonwut S, Woonwong Y, Kedkovid R, Arunorat J, Thanawongnuwech R and Suradhat S 2017. Positive immunomodulatory effects of heterologous DNA vaccine- modified live vaccine, prime-boost immunization, against the highly-pathogenic PRRSV infection. Veterinary Immunology and Immunopathology. 183: 7-15.

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