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Intradermal co-inoculation of codon pair deoptimization (CPD)-attenuated chimeric porcine reproductive and respiratory syndrome virus (PRRSV) with Toll like receptor (TLR) agonists enhanced the protective effects in pigs against heterologous challenge

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ABSTRACT

The objective of this study was to assess protective efficacy of vaccination using CPD-attenuated chimeric PRRSV and Toll like receptor (TLR) agonists (HSP70 c-terminal domain and HSPX) as adjuvants through different inoculation routes. In this study, a chimeric PRRSV composed of two field isolates was synthesized and attenuated by CPD in NSP1 as described in the previous study. The infection of the CPD-attenuated chimeric PRRSV to pigs of 3 weeks-old showed no clinical signs without pathological lesions in necropsy, while it induced improved cross immunity between its parent strains. The TLR agonists were expressed in *E. coli* and purified to be used. In challenge experiment, pigs of 3 weeks-old were vaccinated using the CPD-attenuated chimeric virus with the prepared TLR agonists through intramuscular or intradermal route, following heterologous challenge after 4 weeks of vaccination. In results, intramuscular or intradermal inoculation of the CPD-attenuated chimeric virus demonstrated excellent protective efficacy against heterologous challenges. Importantly, intradermal inoculation with the TLR agonists enhanced protective effects as shown in the significantly increased level of PRRSV-specific IFN-γ-SCs and cytokines in sera, and the significant reduction of pathological lesion and viral load in lung. This study suggested that the intradermal inoculation of CPD-attenuated chimeric PRRSV plus TLR agonists should be more effective for protection of pigs against diverse PRRS field viruses.

1. Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) (the genus Arterivirus, family Arteriviridae, order Nidovirales) is an etiological agent, which is one of the most devastating diseases of pork industry (Keffaber, 1989), since the first outbreak of the virus in United States in 1987. The virus is characterized as reproductive failure in sows and respiratory disease in piglets (Wensvoort et al., 1991), causing the economic loss of approximately $560.32 million per year estimated in the United States (Neumann et al., 2005).

Concurrently, there have been different various measures for prevention and control of the diseases. Among these measures, vaccination with live attenuated strains has been proved to be the most efficient method to protect pigs against field PRRSVs. However, the attenuated strains obtained after long-term serial passages in cell lines have produced concerns on enhanced emergence of genetic/antigenic diverse strains (Cha et al., 2004) and reversion to virulence (Nielsen et al., 2001) in vaccinated animals due to a high probability of mutation. In accordance with past researches (Hanada et al., 2005), inefficient protectivity of live vaccines against field isolates has been caused by the high mutation rate of PRRSV and frequent homologous recombination.

In order to control virulence of various viruses, A computer-based codon pair deoptimization (CPD) technology has been utilized (Coleman et al., 2008; Mueller et al., 2010; Martrus et al., 2013). The rapid viral attenuation concept by CPD is to decrease codon pair bias (CPB), and decreased CPB is associated with inefficiency of the viral gene

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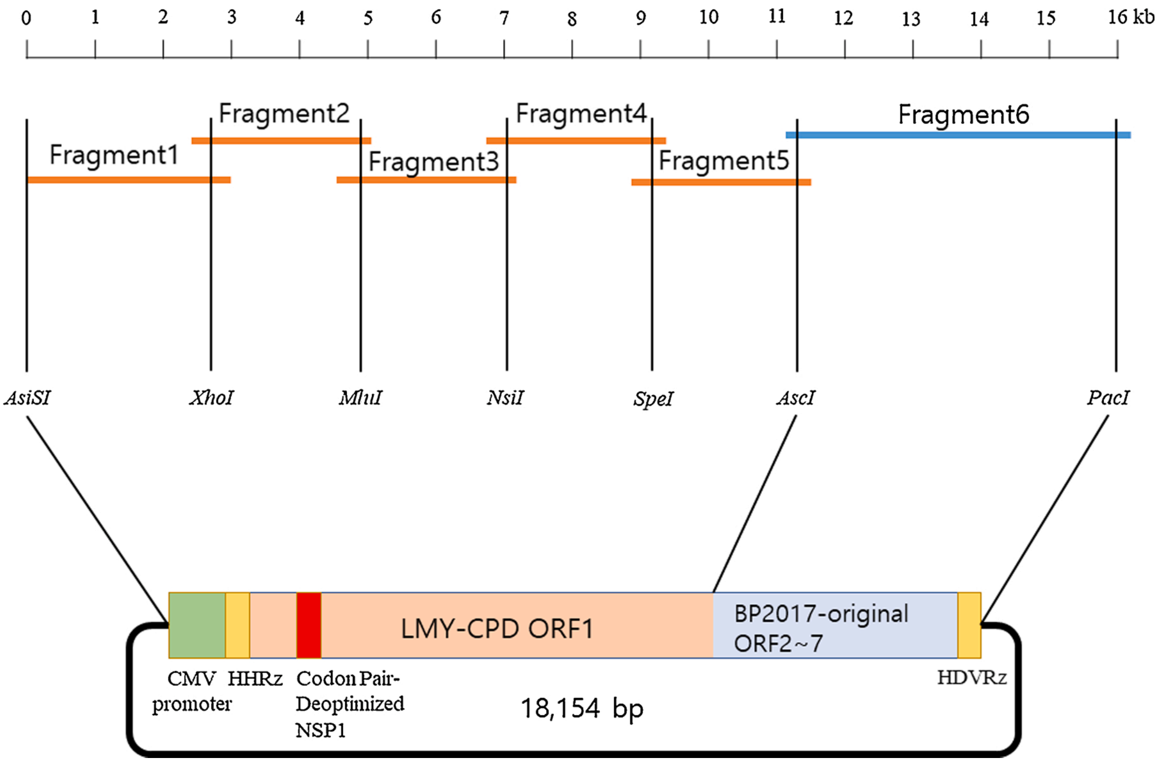

Fig. 1. Design for infectious clones of the recombinant chimeric CPD virus (LMY-BP-CPD).
The LMY-BP-CPD virus was encoded by ORF1 of LMY-CPD with a fragment containing codon-pair deoptimized nsp1, and replaced the structure proteins with ORF2~7 of another field isolates, BP2017-original.

translation, which results in non-reversive attenuation of viral replication (Coleman et al., 2008). Our previous study also successfully applied CPD to NSP1 from Korean isolates in order to attenuate and prove the potential usefulness as live vaccines (Park et al., 2020).

Protective efficacies of live vaccines associated with viral clearance and relief of symptoms varied with wide diversities (Renukaradhya et al., 2015). Among the trials to improve the efficacy, co-inoculation with specific immunostimulant enhanced the immune response against PRRSV in accordance with report. In particular, *Mycobacterium tuberculosis* whole cell lysate (Mtb WCL) was regarded as a potent mucosal adjuvant due to its Th1 biased immune response to PRRS-MLV (Dwivedi et al., 2011). The pigs, which are immunized with PRRS-MLV and Mtb WCL, generated effective cross-protective immunity against heterologous PRRSV and showed an increased frequency of Th cells and an inversely reduced frequency of T-regulatory cells (Dwivedi et al., 2011).

In terms of adjuvant, *Mycobacterium tuberculosis* has various useful proteins associated with Toll like receptors (TLRs). Several mycobacterial TLR2 agonists have already been identified, including certain lipoarabinomannan species, phosphatidyl-myo-inositol mannoside, and lipoproteins (LpqH and LprG) (Jones et al., 2001; Gilleron et al., 2003; Gehring et al., 2004). The TLR agonists have been proved to boost host immune system. They promote dendritic cell maturation and induce proinflammatory cytokine productions (TNF-α, IL-1β, IL-6, and IFN-β) (Jung et al., 2014). Serially, the matured DCs enhance the differentiation, and lead to the activation of cytotoxic T lymphocytes (Jung et al., 2014). Among *Mycobacterium tuberculosis* antigens, the heat shock protein 70 c-terminal (HSP70c) and heat shock protein X (HSPX) have been known as the strongest TLR2 and TLR4 agonist, respectively (Bulut et al., 2005; Jung et al., 2014). Therefore, it is suggested that the heat shock proteins may strongly enhance the protective immunity comparable to Mtb WCL as potent adjuvant of PRRS MLV.

In this study, we generated an CPD-attenuated chimeric virus. For the virus, it contains genomes of two field viruses. Additionally, two heat shock proteins (HSP70c and HSPX) of *Mycobacterium tuberculosis* were expressed and purified from *E coli* vector expression system. Pig studies were conducted to evaluate protective efficacy against heterologous challenge when the CPD-attenuated virus was utilized as a live vaccine with the TLR adjuvants through two different inoculation routes, ID or IM.

2. Material & method

2.1. Viruses and cells

Two CPD-attenuated PRRS viruses, which had been synthesized from type 2 Korean field isolates by applying CPD to NSP1, LMY-CPD (Genbank no. DQ473474) and BP2017-CPD (Genbank no. MK330996) (Park et al., 2020), were used. SNUVR090851 (Genbank no. JN315685) was isolated from lung of PRRS affected pigs in 2010 (Park et al., 2014) and it was used for a heterologous challenge.

MARC-145 cells were used for virus propagation, transfection of the infectious clone, and virus rescue. Porcine alveolar macrophages (PAMs) were obtained by lung lavage from specific-pathogen (PRRSV)-free piglets and, those were used for evaluation of viral attenuation by CPD. Cells were maintained in the appropriate medium as previously described (Park et al., 2020).

2.2. Synthesis and rescue of the CPD-attenuated chimeric virus (LMY-BP-CPD)

A chimeric PRRSV as vaccine strain was synthesized by combining non-structure proteins encoded by ORF1 of LMY-CPD, which was synthesized by CPD in NSP1 of LMY-original, and the structure proteins encoded by ORF2~7 of another field isolates, BP2017-original. The illustration of the chimeric virus is presented in (Fig. 1).

Briefly, in the full-length cDNA clones of LMY-CPD, a specific restriction enzyme site of AscI had been inserted between ORF1 and ORF2.

Table 1
Animal experiment design.

Virulence test of LMY-BP-CPD

Group	Inoculation	Total Number	Necropsy (14 dpi)	Necropsy (28 dpi)
LMY-BP-CPD	LMY-BP-CPD	10	5	5
LMY-CPD	LMY-CPD	10	5	5
LMY-original	LMY-original	10	5	5
BP2017-original	BP2017-original	10	5	5
Negative control		10	5	5

Protective efficacy test of LMY-BP-CPD and the TLR agonists

Group	Inoculation		Adjuvants	Total Number	Challenge	Necropsy (42 dpi)
VC-IDT	LMY-BP-CPD	ID	HSP70c, HSPX	5	SNU 090851	5
VC-ID	LMY-BP-CPD	ID	–	5	SNU 090851	5
VC-IMT	LMY-BP-CPD	IM	HSP70c, HSPX	5	SNU 090851	5
VC-IM	LMY-BP-CPD	IM	–	5	SNU 090851	5
UVC	–			5	SNU 090851	5
UVUC	–			5		5

The procedure had been conducted by de novo synthesis of the fragment, which was component covering the end of ORF1 and start of ORF2. Subsequently, the ORF2~ORF7 of LMY was deleted by AscI and PacI from the full length clone, and replaced with the corresponding part of BP2017-original. The assembled full-length cDNA clones were confirmed by full genomic sequencing.

The full-length cDNA clone composed of the recombinant fragments was transfected into MARC-145 cells as previously described (Ni et al., 2011). After transfection, the cell culture supernatants were collected and serially passaged to MARC-145 cells. An immunofluorescence assay (IFA) using PRRSV specific anti-N protein antibody (SR-30, Rural Technologies Inc., Brookings, SD, USA) was performed in order to confirm the rescue of viruses (Ni et al., 2011).

2.3. TLR agonists preparation

2.3.1. Plasmid construction

The heat shock protein 70 c-terminal (HSP70 c) gene and the heat shock protein X (HSPX) were synthesized according to the sequence of *Mycobacterium tuberculosis* (Genebank no. EU747334.1). They were then inserted respectively into the pET-28a bacterial expression vector (Merk Biosciences, Germany) using an engineered 5′ HindIII site and a 3′ XhoI site. Subsequently, competent BL21 (DE3) cells (Gentaur Molecular, Belgium) were transformed with the plasmids carrying the HSP 70c or HPSX gene. Cells were always grown in presence of kanamycin to ensure the maintenance of all plasmids.

2.3.2. Expression, protein purification, and evaluation of HSP70c and HSPX

Over expression of the target genes was performed as previously described (Jennifer et al., 2012). Briefly, when the transformed BL21 cells grown in LB broth containing kanamycin reached to OD_{600} between 0.8 and 1.2, the protein expression was induced by the addition of 1 mM IPTG. The harvested cell cultures were resuspended in the lysis buffer. Subsequently, the cell lysate was ultrasonicated (20k Hz pulse for 10 min). After the cell lysates were centrifuged, the supernatant was then loaded onto a Ni–NTA affinity column (Qiagen, Netherlands). After the column was washed by gradually increasing concentration of imidazole, the HSP70c and HSPX protein was eluted with 1 M imidazole and dialyzed buffer. For the verification of protein expression, the final elute was subjected to SDS–PAGE (14 % gel) followed by Coomassie staining and Western blot analysis was performed using commercial antibodies against HSP70c (Cat. TS489, Mycobacterium Tuberculosis, Heat Shock Protein 70 Monoclonal Antibody, MyBioSource) and HSPX (Cat. MA1−7065, *Mycobacterium tuberculosis* 16 kDa Monoclonal Antibody(G061), Invitrogen, USA).

Limulus amebocyte lysate testing (BioWhittaker, Walkersville, MD) revealed that the final protein product contained only trace levels of endotoxin (< 1 EU/mL).

2.4. Attenuation of virulence and Protective efficacy of LMY-BP-CPD, inoculation route and TLR agonists

2.4.1. In vitro replication kinetics of LMY-BP-CPD

The capacity of in vitro replication of the rescued chimeric virus was investigated using PAM. The cell was infected with the 4 viruses (LMY-BP-CPD, LMY-CPD, LMY-original, and BP2017-original) at multiplicity of infection of 0.1, respectively. In addition, the culture medium of infected cells was used for viral titration at 0, 1, 2, 3, 4, and 5 days post inoculation (dpi) as described previously (Park et al., 2020).

2.4.2. Pig studies

All information of the animal experiments is presented (Table 1). This study contained two animal experiments according to the purpose. The first experiment focused on the virulence of the CPD-attenuated chimeric virus (LMY-BP-CPD). Total 50 commercial crossbred 3-week-old pigs, which were negative for PRRSV-specific antibodies, were randomly divided into 5 groups (10 pigs per group). All groups had equal sex ratio (5:5). Four groups were inoculated intranasally with 2 mL culture fluid (10^5 $TCID_{50}$/mL) of each from the 4 viruses (LMY-BP-CPD, LMY-CPD, LMY-original, and BP2017-original). The negative group was inoculated with PBS. Following the infection of the viruses, the pigs were monitored weekly to test their physical conditions and scored daily for clinical respiratory disease severity, by using scores ranging from 0 (normal) to 6 (severe dyspnea and abdominal breathing) (Halbur et al., 1995). Meanwhile, rectal temperatures were measured daily at the same time for 28 days post inoculation (dpi) when the study was finished.

The second pig experiment was conducted to evaluate the protective effect of the chimeric virus and TLR agonists depending on inoculation routes. Total 30 commercial crossbred 3-week-old pigs were randomly divided into 6 groups (5 pigs per group). Two groups (VC-IDT and VC-ID) were vaccinated intradermally with 0.5 mL culture fluid ($10^{4.4}$ $TCID_{50}$/mL) of LMY-BP-CPD, while two groups (VC-IMT and VC-IM) were vaccinated intramuscularly with 2 mL culture fluid ($10^{3.8}$ $TCID_{50}$/mL) of LMY-BP-CPD. For the VC-IDT and VC-IMT groups, the TLR agonists were co-inoculated with the virus culture fluid at 10ug/dose respectively. UVC and UVUC group were maintained as unvaccinated. At 28 dpi, all vaccinated groups and UVC group were challenged intranasally with 3 mL of culture fluid containing 10^5 $TCID_{50}$/mL of high virulent virus (SNUVR090851 strain). UVUC group remained as a negative control. All pigs were euthanized by an intravenous injection of sodium pentobarbital and electrocution at 42 dpi (14 dpc).

The methods associated with the studies were approved by the BioPOA Institutional Animal Care and Use Committee.

2.4.3. Quantification of viral RNA

RNA was extracted from serum samples to quantify PRRSV genomic cDNA copy numbers, as previously described (Park et al., 2014). Real-time RT-PCR was designed to detect ORF7 sequences of viruses used in this study using forward primers (5′-AACGGCAAG CAGCAGAAGA-3′ and 5′-GAAGAGAAACCCGGAGAAGC-3′ for

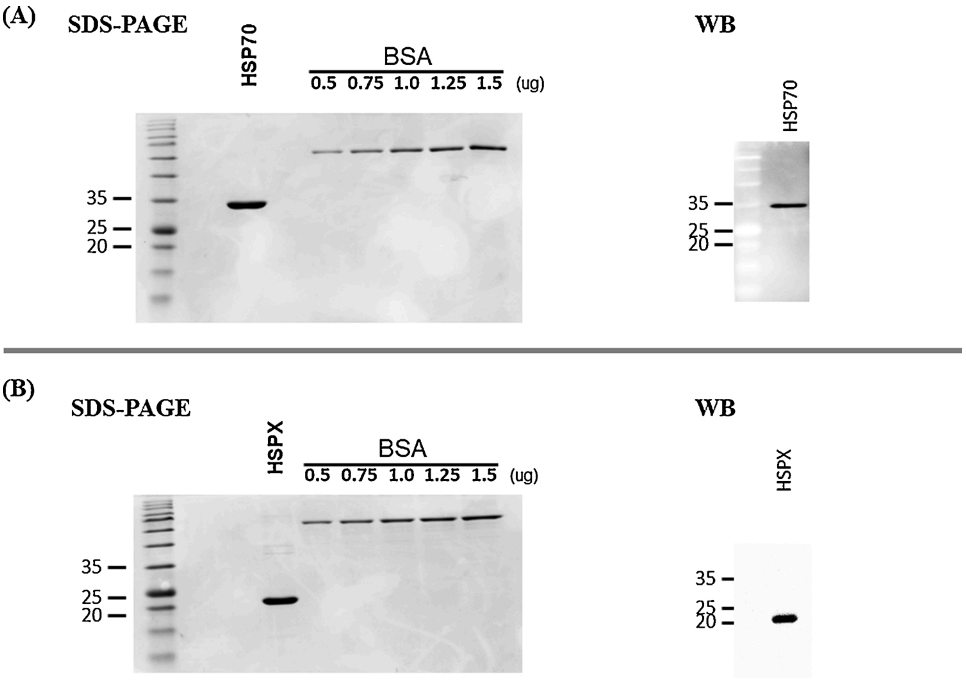

Fig. 2. Confirmation of the purified TLR agonists (HSP70c and HSPX).
(A) The purified HSP70c (31 kDa) and (B) HSPX (20 kDa) were properly placed in SDS-PAGE and detected by the commercial antibody in Western blot.

CPD/original viruses and the challenge virus, respectively) and reverse primers (5′- GTGATGTCTGACATCATCTTCA -3′ and 5′-GAAGAGAA ACCCGGAGAAGC-3′ for CPD/original viruses and the challenge virus, respectively). After standard curves for the primers were obtained, amplification was carried out. The melting curves of amplified products were analyzed to verify the specificity of the PCR, considering a sample with cycle threshold (Ct) of less than 35 cycles (Chung et al., 2005).

2.4.4. *Serology*

Serum samples were collected every week for serological assays. The serum samples were tested, using the commercially available PRRSV enzyme-linked immunosorbent assay (HerdCheck PRRS 3XR™, IDEXX Laboratories Inc.). Serum samples were considered positive for PRRSV antibody if the S/P ratio was greater than 0.4, according to the manufacturer's instructions.

For evaluation of cross neutralizing activity, ELISA based serum neutralization tests were performed at 28 dpi (Virulence test of LMY-BP-CPD) as previously described (Robinson et al., 2015). The procedure was slightly modified as the lab conditions. MARC 145 cells were seeded at 5×10^4 cells/well in 96-well plates, and cultured for 24 h. After heat inactivation for 30 min, the serums were serially diluted in DMEM media without FBS. Quadruple diluted serum was mixed with an equal volume of 10^3 $TCID_{50}$/100 μL PRRSV and incubated for 1 h at 37 °C. The negative control was containing only Marc cells without serum and virus inoculation, and the positive control was including virus-infected Marc cells without serum inoculation. Cells were washed with PBS and subsequently incubated for 24 h. Cells were fixed in 4 % formaldehyde, washed, permeabilized in 0.1 % Triton. After fixation in 4 % formaldehyde and permeabilization in 0.1 % Triton X-100 (Sigma–Aldrich, St. Louis, MO) in PBS, the primary SR-30 antibody and secondary HRP-conjugated goat-anti-mouse IgG (H + L) (Bethyl Laboratories) were performed in order to calculate the infection inhibition percentage. After reaction with TMB peroxidase substrate, the colorization was estimated at 450 nm. Percent inhibition of viral infection at each dilution was calculated as rate with the positive controls after background subtraction of absorbance from the negative controls.

Finally, Fluorescent focus neutralization (FFN) test was also performed with the LMY-BP- CPD virus (Protective efficacy test of LMY-BP-CPD and the TLR agonists). Briefly, the virus (10^3 $TCID_{50}$/100 μL) were incubated for 1 h at 37 °C with serially diluted serum. The plates were transferred to MARC 145 cells and incubated for 24 h. the primary SR-30 antibody and the secondary FITC-conjugated goat-anti-mouse IgG (H + L) (Bethyl Laboratories) were used to detect cellular PRRSV protein. The number of fluorescent foci were counted and endpoints were determined as the highest serum dilution providing a 90 % or greater reduction in infection foci compare to positive controls (only virus infected well without serum inoculation).

2.4.5. *Quantification of cytokines*

The serum samples of the pigs were collected for analyzing the quantification of 9 cytokines induced by infection of LMY-BP-CPD at 3, 7, and 14 dpi. The levels of IFN-α, IFN-γ, IL-12p40, TNF-α, IL-1β, IL-8, IL-4, IL-6, and IL-10 were analyzed by the commercial kit (ProcartaPlex Porcine Cytokine & Chemokine Panel 1, Affymetrix, eBioscience, Austria), according to the manufacturer's instructions. The final antibody magnetic beads were read by a MAGPIX® analyzer (Luminex Corporation) and the cytokine levels were calculated according to standard curves with using xPONENT® 4.2 software (Luminex Corporation).

2.4.6. *Enzyme-linked immunospot (ELISPOT) assay*

Swine PBMCs were isolated from fresh venous blood collected in 5 mM EDTA by centrifugation on a discontinuous gradient using LymphoprepTM (Stemcell Technologies, Vancouver, Canada) as previously described (Chung et al., 2018). The numbers of PRRSV-specific interferon-γ secreting cells (IFN-γ-SC) were determined using peripheral blood mononuclear cells (PBMC), following the procedure in the previous report (Park et al., 2014).

Briefly, the commercial porcine IFN-γ ELISPOT plates (MABTECH) were used to detect IFN-γ secreting cells. 5×10^5 PBMCs were dispensed in the well of plates coated by the porcine IFN-γ capture antibody (10 μg/mL) and stimulated with the inoculated viruses and the challenge virus at an moi. of 0.1 as the recall antigen. After 24 h incubation at 37 °C in a 5 % CO2 atmosphere, cells were removed. The following reaction processes were performed according to the manufacturer's instructions. In all cases, unstimulated cells were included as negative control, whereas phytohaemagglutinin (PHA)-stimulated cells were included as positive control. The number of PRRSV specific IFN-γ-SC were expressed as responding cells in 10^6 PBMCs.

2.4.7. Pathology and immunohistochemistry (IHC)

Macroscopic and microscopic lung lesions were analyzed morphometrically as previously described (Halbur et al., 1995). Briefly, For morphometric analysis of the macroscopic pulmonary lesion score, each lung lobe was assigned a number to reflect the approximate percentage of the volume of the entire lung and the percentage volume from each lobe added to the entire lung score (ranging from 0 to 100 % of the affected lung) as previously described (Halbur et al., 1995).

For morphometric analysis of the microscopic pulmonary lesion score, each slide sections were examined in blinded fashion, and the severity in the interstitial pneumonia were estimated by the score as: 0, no lesions; 1, mild interstitial pneumonia; 2, moderate multifocal interstitial pneumonia; 3, moderate diffuse interstitial pneumonia; and 4, severe interstitial pneumonia.

Immunohistochemistry (IHC) for PRRSV was performed using SR30 antibody and analyzed morphometrically. To calculate PRRSV positive signals, serial tissue sections were analyzed with the NIH Image J 1.43 m Program. Ten fields were selected randomly and the number of positive cells per unit area (0.95 mm2) was determined.

2.5. Statistical analysis

Statistical analyses were carried out using SPSS 16.0 software. The normality which showed the distribution of the examined variables was evaluated by the Shapiro–Wilk test. Group comparison of continuous data (viremia, serum neutralizing antibody, cytokine levels, ELISA s/p ratio, and number of IFN-γ secreting cells) were analyzed by student-t test or one-way ANOVA test following Tukey's adjustment. Lung microscopic score was compared by a non-parametric Mann–Whitney test. All data were expressed as the mean value ± standard deviation (SD). For all of statistical analyses, significance level was set at $P < 0.05$.

3. Results

3.1. Adjuvant HSP70c and HSPX were successfully expressed in E.cOli system

HSP70c and HSPX genes were successfully cloned in pET28a vector respectively. SDS-PAGE analysis revealed that the HSP70c and HSPX proteins containing a histidine tag were purified by Ni-NTA resin and the purified proteins appeared a single band at approximately 31 (HSP70c) and 20 (HSPX) kDa on SDS-PAGE. In addition, Western blot assay demonstrated that the purified proteins were detected by the commercial antibodies against the *Mycobacterium tuberculosis* heat shock proteins, HSP70c and HSPX (Fig. 2).

3.2. The CPD-attenuated chimeric PRRSV (LMY-BP-CPD) showed similar growth pattern with its parent attenuated strain (LMY-CPD) in vitro

The LMY-BP-CPD was successfully rescued from MARC 145 cell. PRRSV-specific CPE featured with clumping and apoptosis in the viral genome-transfected MARC 145 cells was observed and PRRSV replication was confirmed by the immunofluorescence assay detecting the viral protein. Nucleotide sequences of NSP1 of the virus were changed as designed by the computer program for codon pair de-optimization. It has exactly the same the CPB (codon pair bias) level (-0.2393) with the previously codon pair deoptimized LMY strain (LMY-CPD) (Park et al., 2020).

In the case of viral replication in PAM cells, LMY-BP-CPD showed similar growth pattern with LMY-CPD. It reached to the peak of viral titer at 1 dpi and decreased till 5 dpi. The viral titers of LMY-CPD (insert range of viral titers) and LMY-BP-CPD (insert range of viral titers) were significantly (p < 0.05) lower than those of the LMY-original (insert range of viral titers) and BP2017-original (insert range of viral titers) with unmodified NSP1 gene at 2, 3, 4, and 5 dpi. There was no significant difference between LMY-CPD and LMY-BP-CPD (Fig. 3A).

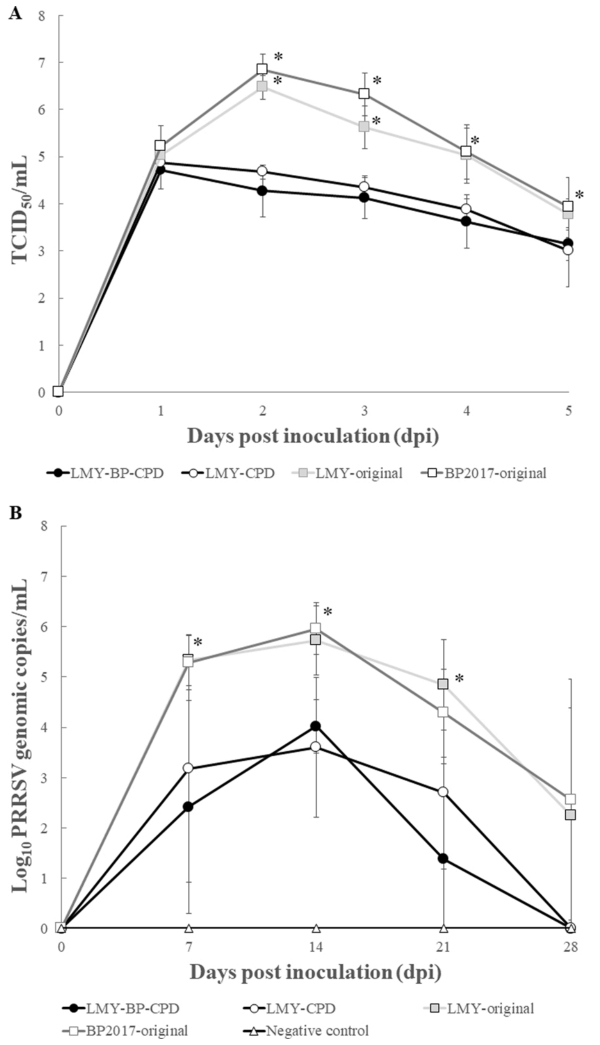

Fig. 3. Comparison of replication kinetics of original and CPD viruses. (A) In the case of viral replication in PAM cells, the viral titers of LMY-BP-CPD (●) and LMY-CPD (○) were significantly lower than that of the LMY-original (▨) and BP2017-original (□) at 2, 3, 4, and 5 dpi. Likewise, (B) In the pig study, the viremia of LMY-BP-CPD (●) and LMY-CPD (○) were significantly lower than that of the LMY-original (▨), BP2017-original (□), and Negative control (△) at 7, 14, and 21 dpi. Significant difference ($P < 0.05$) was indicated by '*'.

3.3. Virulence test of LMY-BP-CPD : 1st animal experiment

3.3.1. The pigs infected with LMY-BP-CPD showed normal rectal temperature and weigh gain

The pigs infected by the two original viruses (LMY-original and BP2017-original) showed mild clinical signs with moderate dyspnea and diarrhea for 14 days, while not observed in pigs infected with CPD viruses (LMY-CPD and LMY-BP-CPD). For the experiment period, there was no pig with rectal temperature over 40.5 °C. Besides, no significant difference of ADWG was observed between the groups for entire experiment.

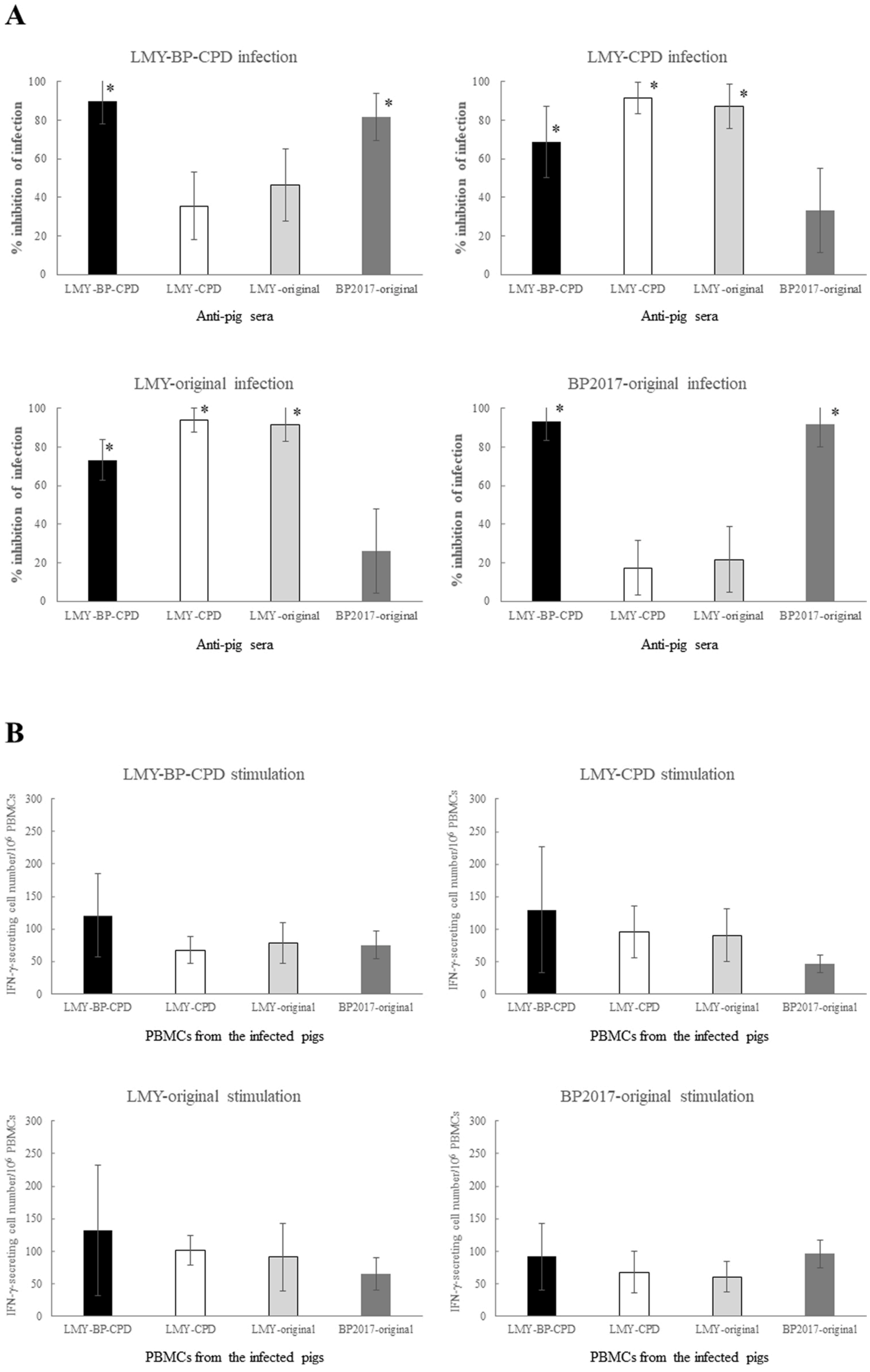

Fig. 4. Cross-immune responses between the original and CPD viruses.

(A) Cross-neutralizing activity of serum sample from pigs inoculated with original or CPD viruses. The pigs infected by LMY-BP-CPD (■) had broad cross-neutralizing activity against the parent strains. Significant difference ($P < 0.05$) was indicated by '*'. (B) The mean numbers of PRRSV-specific IFN-γ-SCs from PBMCs of pigs inoculated with original or CPD viruses. There were no significant differences between the groups.

3.3.2. LMY-BP-CPD replicated at significantly lower level than its parent strains

Viral genes of the inoculated viruses were detected in sera of pigs from 7 dpi to 28 dpi. However, numbers of genomic copies (insert range of genomic copies) in sera of pigs infected with CPD viruses (LMY-CPD and LMY-BP-CPD) were significantly ($p < 0.05$) lower than those (insert range of genomic copies) of pigs infected with original viruses (LMY-original and BP2017-original) at 7, 14, and 21 dpi, by decreasing up to 1,000 times when compared to the original viruses (Fig. 3B).

3.3.3. LMY-BP-CPD induced antibodies with broad neutralization activity

All virus-inoculated pigs were sero-converted from 14 dpi. There was no significant difference in average of ELISA s/p ratio between the groups throughout the experiment.

However, the sera from pigs infected with LMY variants (LMY-BP-CPD, LMY-CPD, and LMY-original) had significantly ($p < 0.05$) higher virus neutralization activity against LMY-CPD and LMY-original infection than those with BP2017-original in an ELISA-based neutralization assay on MARC 145 cells (Fig. 4A). At the serum dilution of four times,

Table 2
Pathological examination of the virulence test.

Attenuation of chimeric CPD PRRSV						
	Dpc	Groups LMY-BP-CPD	LMY-CPD	LMY-original	BP2017-original	Negative control
Macroscopic lung lesion score	14	2 ± 4.4	2 ± 4.4	32 ± 8.3*	28 ± 8.3*	0 ± 0
	28	0 ± 0	0 ± 0	2 ± 4.4	2 ± 4.4	0 ± 0
Microscopic lung lesion Score	14	0.2 ± 0.2	0.5 ± 0.5	2.2 ± 0.2*	1.8 ± 0.5*	0 ± 0
	28	0 ± 0	0.2 ± 0.1	0.4 ± 0.3	0.3 ± 0.2	0 ± 0
Lung antigen score	14	4.6 ± 1.8	7.6 ± 1.9	27 ± 9.4*	21.8 ± 9*	0 ± 0
	28	0 ± 0	2.6 ± 2.6	5.2 ± 6.8	4.2 ± 2.7	0 ± 0

Different letter (*) indicate significant ($P < 0.05$) difference among groups.

they had average inhibition percentage of 68.4 ± 18 % (LMY-BP-CPD group), 91.4 ± 8% (LMY-CPD group), 87 ± 11 % (LMY-original group), and 33 ± 21 % (BP2017-original group) against LMY-CPD infection, and showed 73.1 ± 10 % (LMY-BP-CPD group), 93.8 ± 6% (LMY-CPD group), 91.6 ± 8% (LMY-original group), and 25.7 ± 21 % (BP2017-original group) against LMY-original infection. While the neutralization activity against LMY-BP-CPD and BP2017-original was significantly higher in LMY-BP-CPD group and BP2017 group. The inhibition percentage was 89.9 ± 11 % (LMY-BP-CPD group), 35.3 ± 17 % (LMY-CPD group), 46.2 ± 18 % (LMY-original group), and 81.7 ± 12 % (BP2017-original group) against LMY-BP-CPD infection. It was equivalent with the inhibition activity of 92.8 ± 9% (LMY-BP-CPD group), 17.2 ± 13 % (LMY-CPD group), 21.5 ± 17 % (LMY-original group), and 91.6 ± 11 % (BP2017-original group) against BP2017-original infection (Fig. 4A).

3.3.4. LMY-BP-CPD induced PRRSV-specific interferon-γ-secreting cells

After stimulation with homologous and heterologous virus, mean numbers of PRRSV-specific IFN-γ-SCs from PBMCs of CPD and original virus infected pigs were estimated at 28 dpi without significant difference among the groups (Fig. 4B).

3.3.5. The pigs infected with LMY-BP-CPD showed significantly lower macroscopic and microscopic lesion than the pigs with its parent strains

The groups infected by original viruses (LMY-original and BP2017-original) showed macroscopic lung lesions characterized by multifocal, tan-mottled areas, with irregular and indistinct borders at 14 dpi. The macroscopic lung lesion scores of LMY-BP-CPD (2 ± 4.4) or LMY-CPD (2 ± 4.4) were significantly ($P < 0.05$) lower than those of LMY-original (32±8.3) or BP2017-original (28±8.3).

Microscopic lung lesions associated with PRRSV were observed in pigs infected with the original viruses (LMY-original and BP2017-original), while there were no clear pathological lesions in those (LMY-BP-CPD and LMY-CPD) infected with the CPD viruses and negative control. Microscopic lung lesion scores of LMY-BP-CPD (0.2 ± 0.2) or LMY-CPD (0.5 ± 0.5) were significantly ($P < 0.05$) lower than those of LMY-original (2.2±0.2) or BP2017-original (1.8±0.5) at 14 dpi (Table 2). There was no significant difference among groups inoculated with the two CPD viruses and negative control. The lesions were hardly found at 28 dpi among all groups.

PRRSV antigen was detected by immunohistochemistry in the lungs of all pigs except negative control group at 14 dpi. The mean number of PRRSV-positive cells per unit area of lung was significantly ($P < 0.05$) lower in LMY-BP-CPD (4.6±1.8) or LMY-CPD (7.6±1.9) inoculated pigs than in LMY-original (27±9.4) or BP2017-original (21.8±9) inoculated pigs at 14 dpi (Table 2). There was no significant difference among the groups at 28 dpi.

3.4. Protective efficacy test of LMY-BP-CPD and the TLR agonists routes: 2nd animal experiment

3.4.1. The vaccination by LMY-BP-CPD successfully protected pigs from fever and wasting regardless inoculation routes or adjuvants

Throughout this study, all vaccinated groups (VC-IDT, VD-ID, VC-IMT, and VC-IM) and UVUC did not show clinical signs associated with PRRS. After challenge, mean rectal temperatures in the VC-IDT (39 ± 0.5), VC-ID (39.2 ± 0.4), VC-IMT (39 ± 0.3), and VC-IM (39.3 ± 0.6) were significantly ($P < 0.05$) lower than in the UVC (40.5±0.4) at 3 dpc (Table 3).

The ADWG of all vaccinated groups (VC-IDT, VD-ID, VC-IMT, and VC-IM) and UVUC was significantly ($P < 0.05$) higher than that of the UVC from 7 to 14 dpc. There was no significant difference between the vaccinated groups and UVUC over 0 dpc to 14 dpc (Table 3).

Table 3
Clinical and pathological examination of the challenge experiment.

The protective efficacy of LMY-BP-CPD and the TLR agonists							
	Dpc	Groups VC-IDT	VC-ID	VC-IMT	VC-IM	UVC	UVUC
Rectal temperature	3	39.5 ± 0.5	39.2 ± 0.4	39 ± 0.3	39.3 ± 0.6	40.5 ± 0.4*	39.4 ± 0.4
ADWG[a]	7	540 ± 302	534 ± 120	560 ± 216	520 ± 188	400 ± 186	542 ± 119
	14	657 ± 119	630 ± 150	600 ± 63	605 ± 73	328 ± 139*	700 ± 216
Macroscopic lung lesion score	14	16 ± 11†‡	22 ± 8.3†	24 ± 8.9†	28 ± 8.3†	58 ± 13*	0 ± 0‡
Microscopic lung lesion Score	14	0.4 ± 0.3	1.1 ± 0.3†	1.2 ± 0.5†	1.2 ± 0.5†	3 ± 0.4*	0 ± 0
Lung antigen score	14	2.6 ± 0.5	8.9 ± 4.8†	8.3 ± 6.1†	9.5 ± 4.4†	31.1 ± 3.4*	0 ± 0

Different letters (*, †, and ‡) indicate significant ($P < 0.05$) difference among groups.

[a] Average daily weight gain.

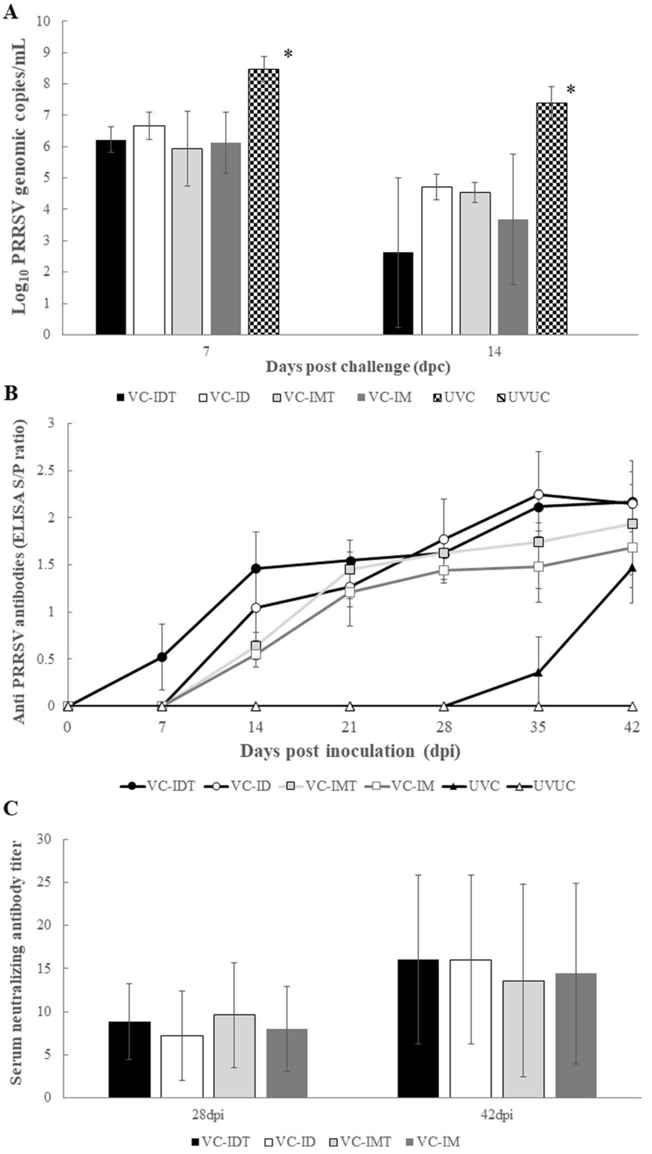

Fig. 5. Protection by vaccination with LMY-BP-CPD in pigs against the heterologous challenge.

(A) Genomic copy number of the challenge virus in serum samples from pigs at 7 and 14 dpc. '*' indicates significant difference ($P < 0.05$) between the UVC group and vaccinated groups.

(B) PRRSV-specific antibody titer (ELISA S/P ratio). (C) Serum neutralizing antibody titer using LMY-BP-CPD virus.

3.4.2. *All vaccinated pigs showed significantly lower viral copies in serum than the non-vaccinated challenged control*

Viral genes of LMY-BP-CPD were detected in sera of all vaccinated groups (VC-IDT, VD-ID, VC-IMT, and VC-IM) from 7 dpi to 21 dpi. However, any genome of PRRSV was not detected in the serum samples of all pigs on the challenge day (28 dpi). After challenge, the number of challenging virus genomic copies was significantly lower ($P < 0.05$) in the vaccinated groups (VC-IDT, VD-ID, VC-IMT, and VC-IM) than UVC (Fig. 5A) at 7 and 14 dpc. No PRRSV RNA was detected from UVUC throughout the experiment.

3.4.3. *The vaccination induced the neutralizing antibodies*

Sero-conversion was observed in all vaccinated groups (VC-IDT, VD-ID, VC-IMT, and VC-IM) from 14 dpi (Fig. 5B). The neutralizing antibodies (NAs) titers against each of homologous viruses were detected at low level (insert range of VN titers) in the vaccinated groups at 28 dpi (0 dpc) and were increased at 42 dpi (14 dpc) (Fig. 5C). There was no significant difference among groups.

3.4.4. *The pigs intradermally inoculated with the adjuvant showed strong proinflammatory cytokine reaction*

Nine cytokines (IFN-α, IFN-γ, IL-12p40, TNF-α, IL-1β, IL-8, IL-4, IL-6 and IL-10) were examined from sera at 3, 7, and 14 dpi. In terms of initial response by vaccination, there was critical variation of specific cytokine levels among the tested groups. IFN-α, IL-8, and IL-12 level of VC-IDT was significantly ($P < 0.05$) higher than that of the other groups (VC-ID, VC-IMT, VC-IM, UVC, and UVUC) at 3dpi (Fig. 6A). The levels of the other cytokines examined in this study were not significantly different among groups (data was not shown).

3.4.5. *The pigs intradermally inoculated with the adjuvant showed strong PRRSV-specific interferon-γ response through the experiment*

The number of vaccine virus-specific IFN-γ-SCs of VC-IDT was significantly higher than that of the other groups (VC-ID, VC-IMT, VC-IM, UVC, and UVUC) on 7, 14, 21, 28 dpi (0 dpc), and 42 dpi (14dpc). After challenge, the number of vaccine virus-specific IFN-γ-SCs was significantly increased in all vaccinated groups to 42dpi (14 dpc). (Fig. 6B). The number of PRRSV-specific IFN-γ-SCs by stimulation with the challenge virus was similar to that by stimulation with the vaccine strain (data not shown). The number of PRRSV specific IFN-γ-SCs remained at basal levels (< 10 cells/10^6 PBMCs) in UVC and UVUC.

3.4.6. *The vaccination reduced pathologic lesion in tissue after challenge*

The mean macroscopic lung lesion scores of VC-IDT (16 ± 11), VC-ID (22 ± 8.3), IC-IMT (24 ± 8.9), and VC-IM(28 ± 8.3) and UVUC were significantly lower ($P < 0.05$) than those of UVC (58±13) at necropsy. No macroscopic lung lesions were observed in UVUC (Table 3). Microscopic lung lesions were also observed in all pigs except UVUC ; moderate interstitial pneumonia lesion scores of all vaccinated groups (VC-IDT, VC-ID, IC-IMT, and VC-IM) were significantly ($P < 0.05$) lower than that of UVC at necropsy. Among the vaccinated groups, pigs vaccinated intradermally with TLR agonists (VC-IDT) showed significantly ($P < 0.05$) lower microscopic lesion score (0.4±0.3) than those with VC-ID (1.1±0.3), VC-IMT (1.2±0.5), and VC-IM (1.2±0.5) (Table 3).

PRRSV antigen was detected by immunohistochemistry in the lungs of all pigs except UVUC. The mean numbers of PRRSV-positive cells per unit area of lung in all vaccinated groups (VC-IDT, VC-ID, IC-IMT, and VC-IM) were significantly ($P < 0.05$) lower than that in UVC at necropsy. Meanwhile, the pigs vaccinated intradermally with TLR agonists (VC-IDT) showed significantly ($p<0.05$) lower number of PRRSV-positive cells in lung (2.6±0.5) than those with VC-ID (8.9±4.8), VC-IMT (8.3±6.1), and VC-IM (9.5±4.4) (Table 3).

4. Discussion

In our previous study (Park et al., 2020), we successfully attenuated Korean field isolates by applying CPD technology to NSP1 of PRRSV genomes and demonstrated the usefulness of the technology to generate live vaccine. However, due to continuous diversification of PRRSV, it is crucial to improve the cross-protective efficacy of the vaccine, in order to minimize vaccine failure in the field. In this study, we attempted to enhance the heterologous protective effect through mixing genetic fragments and modulating immune responses

Firstly, we have constructed the CPD attenuated chimeric PRRSV (LMY-BP-CPD) by reverse genetics, which consists of two Korean field isolates genome. It retained the ORF1 encoding non-structural proteins of LMY strain and the ORF2−7 encoding structural proteins of BP2017-2. To improve the low cross-protective effect in PRRSV-infected pigs against heterologous field isolates, multiple structural protein genes shuffling was attempted to produce chimeric viruses (Tian et al., 2017; Zhou et al., 2012). Throughout this shuffling, heterologous protection

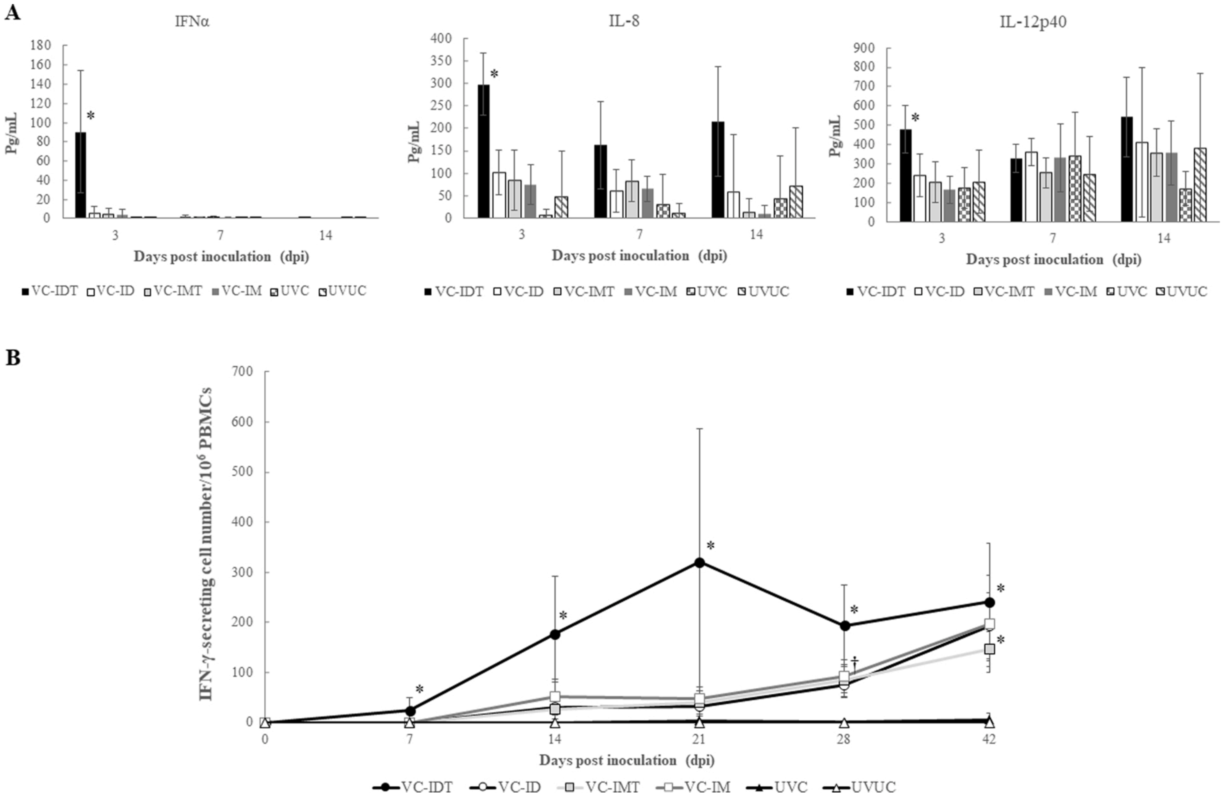

Fig. 6. Immune responses depending on inoculation route.
(A) Cytokine levels of serum from pigs depending on inoculation route at 3, 7, and 14 dpi. (B) The mean numbers of vaccine strain-specific IFN-γ-SCs from PBMCs of pigs. '*' indicates significant difference ($P < 0.05$) between the VC-IDT and the others.

was improved. Studies reported that neutralization of antibodies were induced by the structural proteins (GP2, GP3, GP4, GP5, and M) (Popescu et al., 2017; Trible et al., 2015) as well as non-structural protein (ORF1a) (Leng et al., 2017) of PRRSV. Some studies also identified T lymphocyte epitopes in several PRRSV proteins in structural proteins (GP2, GP5, M, and N) (Bautista et al., 1999; Diaz et al., 2009) and non-structural proteins (NSP2, NSP9, and NSP10) (Burgara-Estrella et al., 2013; Parida et al., 2012). Therefore, it is reasonable to expect that the chimeric virus constructed in our study would contain improved cross-protective effect.

CPD was successfully applied to NSP1 of the chimeric virus to reduce virulence. It was confirmed that the replication capability and pathogenicity of LMY-BP-CPD were significantly reduced in PAM cells and pigs, respectively. On the other hand, the level of immune response (PRRSV-specific antibodies and IFNγ-SCs) was comparable to that by original viruses (LMY-original and BP2017-2-original) and LMY-CPD, as observed in the previous study (Park et al., 2020). In terms of cross-reactivity, as shown in this study, the LMY-BP-CPD showed high level of cross-neutralizing antibodies activity against both original viruses, LMY and BP2017-2, while the two viruses showed low level of cross-neutralizing activity against each other. Besides, PBMCs from the CPD chimeric virus-infected pigs showed no significant difference in the number IFNγ-SCs regardless of PRRSV strain for stimulation. Therefore, it was anticipated that vaccination of LMY-BP-CPD may induce an improved cross-protective immunity.

On the basis on the attenuated virulence and the capability of inducing PRRSV-specific immune response, the possible protective effect of LMY-BP-CPD against heterologous challenge was enhanced by utilizing TLR agonists and different inoculation routes (IM and ID). Basically, it was confirmed that vaccination of LMY-BP-CPD induced PRRSV-specific protective immunity. The all vaccinated groups significantly improved clinical score, growth performance and PRRSV-associated pathological lesion. These improvements indicated protection of pigs from PRRS by the heterologous strain, which shares 85.5 % nucleotide identity for ORF5 with the vaccine viruses.

However, it was noted that there were dramatical enhancements of immune responses and protective effects only in pigs for intradermal inoculation along with TLR agonists (VC-IDT group) The VC-IDT group had significantly higher cytokine responses (IFN-α, IL-8, and IL-12) in sera collected at 3 dpi and number of IFN-γ-SCs during entire experiment than other groups. Additionally, the enhanced IFN-γ response of VC-IDT may be positively correlated with IFN-α levels in the serum (not statistical). As previously described, the induction of either a Th1 or Th2 immune response can be controlled by the antigen-presenting cells and their cytokines, especially IFN-α (Brinkmann et al., 1993). IFN-α is not only involved in innate immunity, but also participate in various stages of the activation of adaptive immunity (McNab et al., 2015). DCs are potent producers of IL-12, which is crucial for driving Th 1 responses during some viral infections, and important for IFN-γ production by T cells and NK cells (Gautier et al., 2005). Therefore, it was suggested that the high level of IFN-α and IL-12 in VC-IDT at early time of vaccination may be related to the significant increase in IFN-γ production by PBMCs. The TLR agonists used in this study were known as a method to induce specific cytokine responses. In case of HSP70, it reportedly produced considerable level of IFN-α, IL-6, TNF-α, and IL-12 in DCs, and also lead to DCs activation to differentiate naïve T cells into effector T cells, which produce IFN-γ under control of TRIF and MyD88 (Kim et al., 2015b). Additionally, HSPX was involved in regulation of the Th1/Th2 immune response in DCs and increased IL-17, TGF-β, IL-12 and IFN-γ production (Kim et al., 2015a). Despite allogenic discordance with reported cytokines, it was definite fact that the TLR agonists have induced high level of IFN-γ in VC-IDT

The enhanced immune responses were not observed in VC-IMT. Despite of utilizing the same TLR agonists, their immune responses were similar to those of other vaccine groups (VC-ID and VC-IM) without TLR agonists. Therefore, it was suspected that the enhanced immune response by the TLR agonists might be resulted from the antigen delivery system in inoculation site. The intramuscular inoculation induces prompt systemic immune responses due to faster rates of adsorption than other inoculation sites. In comparison, the intradermal inoculation leads to immune responses, which are directly linked to Langerhans cells as a main antigen presenting cell. In the activation of Langerhans cells by foreign antigens, the TLR agonists in the vaccine might be potential to elicit the synergistic effect of innate and adaptive immune responses. Superior efficacy according to inoculation route is also supported by the previous reports that were conducted with the commercial live attenuated vaccine. Under field conditions to naturally expose the pigs to a heterologous virulent PRRSV, the frequency of IFNγ SC and $CD3^{-}CD8\alpha^{+}$ NK cells in PBMC were significantly increased in the intradermally vaccinated pigs than in the intramuscularly vaccinated pigs and controls (Martelli et al., 2009). In challenge experiment utilizing HP-PRRSV, intradermally vaccinated pigs had significantly lower IL-10 levels and higher IFN-γ-SC than that of intramuscularly vaccinated pigs. It strongly implied that the intradermal route may represent an alternative to improve vaccine efficacy (Madapong et al., 2020). The prompt and intensive innate immune response may be a key factor to prevent PRRSV. Poor anti-viral cytokine levels (especially, IFN-α and TNF-α) and delayed specific immune responses were usually reported in PRRSV infection cases, so that it needs over 4 weeks to induce meaningful protective immune response (Van Reeth et al., 1999; Dwivedi et al., 2012, and Meier et al., 2003). Similar with their parents, current MLV vaccines demand some weeks for the efficient immunity. However, there are normally only a few days between period of maternal antibody interference and timing of infection by field strains, the vaccine efficacy cannot be expected to control the field situation. In this study, direct exposure of adjuvants to immune cells can boost strong cytokine response and lead to the expedite PRRSV-specific cell-mediated immune response. It suggested that the vaccine inducing the prompt and strong immunity can be the game changer to improve the field situation.

The increased immune responses by intradermal inoculation route and TLR agonists were positively reflected on protective effects against heterologous challenge. Viremia of the challenge virus was significantly reduced in all vaccinated groups compared with the non-vaccinated challenged group (UVC). Interestingly, any viral antigen has not been detected in the serum and necropsied tissues of two pigs from VC-IDT at 14 dpc. Additionally, the group even had lower average level of viral loads than the level of other vaccine groups. Clinical score, pathological lung lesion score, and viral antigen of lung were also significantly reduced in all vaccinated groups. Especially, VC-IDT showed significantly lower interstitial pneumonia lesion score and PRRSV-positive cell number in lung than the other vaccinated groups (VC-ID, VC-IMT, and VC-IM). These reduction of viral loads and PRRSV-specific lesion in VC-IDT suggested that the TLR agonists and intradermal inoculation may contribute to prompt viral clearance from pigs challenged with heterologous strain.

In conclusion, the chimeric virus recombined with two field isolates was successfully attenuated by CPD and induced protective immunity against heterologous challenge with the possibly improved neutralizing activity against diverse field viruses. More importantly, the potential TLR agonists, the HSP70 c-terminal and HSPX of *Mycobacterium tuberculosis* and intradermal inoculation demonstrated enhancement of heterologous protection, suggesting that the vaccination of the CPD chimeric virus plus TLR agonists by intradermal inoculation may provide highly efficient protection for pigs in the field.

Declaration of Competing Interest

The authors report no declarations of interest.

Acknowledgments

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