**Porcine GPX1 enhances GP5-based DNA vaccination against Porcine
Reproductive and Respiratory Syndrome Virus**

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Abstract

Porcine reproductive and respiratory syndrome virus (PRRSV) has been causing huge economic losses to the swine industry worldwide. Commercial PRRSV vaccines including killed and modified live ones are available. However the protective efficacy of these vaccines is incomplete. Thus, it is in urgent need to develop safer and more effective PRRSV vaccines. In this study, we constructed a recombinant plasmid co-expressing porcine glutathione peroxidase-1 (GPX1) and the envelope glycoprotein (GP5) encoding-gene of PRRSV (pcDNA3.1-GPX1-LSynORF5), and investigated the immune response induced following inoculation of mice and piglets. Significantly enhanced GP5-specific antibody, PRRSV-specific neutralizing antibody, IFN- γ level, as well as lymphocyte proliferation response, were induced in mice and pigs immunized with the DNA construct encoding GPX1 and GP5 compared with those inoculated with a construct encoding PRRSV GP5 only (pcDNA3.1-SynORF5). The enhanced cellular immune response in pigs induced by pcDNA3.1-GPX1-LSynORF5 was comparable to that induced by the attenuated virus vaccine JXA1-R, although the humoral immune response induced by the plasmid was much lower than the response induced by JXA1-R. Following the challenge with highly pathogenic PRRSV, less-severe clinical signs and rectal temperatures were observed in pigs immunized with the GPX1-GP5 construct compared with the control group. However, the viraemia of groups immunized with plasmid was more severe than that inoculated with JXA1-R, and it is likely that this could be attributed to the poor humoral response induced by the GPX1-GP5 construct. These results demonstrated that inclusion of GPX1 in a

PRRSV DNA vaccine leads to an adjuvant effect, enhancing the humoral and cellular responses following vaccination.

Keywords

PRRSV; DNA vaccine; GPX1; Immune response

Abbreviations

dpi: days post infection dpc: days post challenge i.m.: intramuscular injection RNA: ribonucleic acid RT-qPCR: quantitative reverse transcription polymerase chain reaction CMI: cell-mediated immune GPX1: glutathione peroxidase-1 PBMCs: peripheral blood mononuclear cells Se: Selenium SI: stimulation index

1. Introduction

Porcine reproductive and respiratory syndrome (PRRS) is characterized by severe reproductive failure in sows, and respiratory distress in piglets and growing pigs (Du et al., 2013). Since first being reported in the United States in 1987 and in Europe in 1990, PRRS has been gaining increased attention because of its large-scale outbreaks and detrimental impact on the global swine industry (Wensvoort et al., 1991). The causative agent, porcine reproductive and respiratory syndrome virus (PRRSV), has a single-stranded, positive-sense RNA genome with a size of approximately 15 kb, which contains nine open reading frames (ORFs). GP5, the major envelope glycoprotein, plays important roles in virus infection, cell-binding and virus adsorption. GP5 is the key immunogenic protein, which can induce humoral and cellular immune responses. (Hou et al., 2008, Jiang et al., 2006, Jiang et al., 2007, Qiu et al., 2005 and Shen et al., 2007). In our previous studies, the modified GP5 vaccine induced enhanced neutralizing antibody and cellular immune responses, compared with the native GP5 (Fang et al., 2006 and Li et al., 2009). Therefore, we aimed to construct more effective PRRSV DNA vaccines on the basis of SynORF5 gene.

Selenium (Se) is an essential micronutrient for humans and animals. Adequate Se is essential for immune function and can protect the immune system from oxidative damage (Hoffmann, 2007). It regulates function of the immune system through humoral, cellular and innate immunity (Hoffmann and Berry, 2008, Huang et al., 2012a, Huang et al., 2012b and Kiremidjian-Schumacher et al., 1992) and its

important biological functions have been attributed largely to its presence in selenoproteins as the 21st amino acid, selenocysteine. The earliest evidence that Se is involved in immune function came in 1959 with the observation that dogs injected with ⁷⁵Se incorporated the isotope into a leukocyte protein; this protein is now known to be cytoplasmic glutathione peroxidase (cGPX) (McKenzie et al., 1998), one of the many selenoproteins in the body. Moreover, mouse models have revealed that low Se intake or genetic deletion of glutathione peroxidase-1 (GPX1) leads to increase virulence of coxsackievirus B3 (Beck et al., 1998). The same study demonstrated that Se deficiency influences various components of antiviral immune responses as well. Other studies have found poor antibody response in Se-deficient animals (Kukreja and Khan, 1998). The positive effects of GPX1 selenoprotein on immune system suggest that it can be used as a molecular adjuvant.

In this study we studied the potential adjuvant effect of GPX1 on DNA vaccines. We successfully generated a recombinant plasmid simultaneously expressing PRRSV GP5 and swine GPX1 and used it to vaccinate mice and pigs. GPX1 co-expression enhanced humoral and cellular responses in both species and vaccinated pigs were partially protected against a challenge by highly virulent PRRSV strain.

2. Material and methods

2.1. Plasmids, cells and virus

Plasmid pcDNA3.1-SynORF5, based on the native ORF5 gene of highly pathogenic

PRRSV strain JSKM (GenBank accession number HQ832104) (Li et al., 2009), pcDNA3.1 (DingGuo ChangSheng, Beijing, China), Vero cells, and Marc-145 cells (China Center for Type Cultural Collection, Wuhan, China) were maintained in our laboratory.

HP-PRRSV strain JSKM, isolated from the lungs of a pig infected with the “high fever” syndrome in Jiangsu Province, was propagated and titrated in Marc-145 cells, as previously described (Lewis et al., 2010).

2.2. PCR amplification and sequencing of GPX1 and LSynORF5 genes

Primers (Table 1) were designed for amplifying GPX1 based on the published gene sequence of the porcine GPX1 gene (GenBank accession number NM_214201.1).

Primers (Table 1) were designed for amplifying LSynORF5 (Linker (GPGP) + SynORF5) based on the sequence of pcDNA3.1-SynORF5, sequenced by Intrivogen Trading, Shanghai and analyzed by BLAST. All of the primers were synthesized by TAKARA, Dalian. PCR reactions for amplifying GPX1 and LSynORF5 with *Taq* DNA polymerase (TAKARA, Dalian, China) were run in a PCR thermal cycler (TAKARA, Dalian, China) with the following program: denaturation at 95°C for 5 min, 30 cycles of denaturation at 95°C for 1 min, annealing at 57°C or 55°C for 1 min, and extension at 72°C for 1 min and ended with a final extension of 10 min at 72°C.

2.3. Construction of pcDNA3.1-GPX1-LSynORF5 plasmids

The cloned products of GPX1 and LSynORF5 were cloned into the pMD¹⁹-T vector (TAKARA, Dalian, China) and then sequenced. The GPX1 PCR products and pcDNA3.1 were digested individually with *Kpn* I/*Eco*R I and ligated using T4 DNA ligase (TAKARA, Dalian, China). The successfully constructed plasmid and LSynORF5 PCR products were then digested with *Eco*R I/*Xho* I, and ligated using T4 DNA ligase. The recombinant plasmids were purified by AxyPrep Plasmid Miniprep Kit (Axygen Biosciences, Zhejiang, China), and confirmed the accuracy followed by digesting with three pairs of restriction enzyme, which included *Kpn* I/*Eco*R I, *Eco*R I/*Xho* I and *Kpn* I/*Xho* I. All of the restriction enzymes were purchased from TAKARA, Dalian.

2.4. Transfection and Western Blotting

Vero cells were seeded at a concentration of 2.5×10^4 cells/well into 6-well tissue culture plate until the cells reached approximately 70 - 80% confluence. Transfection was performed with Lipofectamine 2000 reagent (Invitrogen, USA) in accordance with the manufacturer's protocol. For Western Blot analysis, the transfected cells were collected at 48 h after transfection, and cell lysates were separated by 10% SDS-PAGE (Absin, Shanghai, China) and transferred to a nitrocellulose membrane. The membrane was blocked with 5% fat-free milk in phosphate-buffered saline (PBS) and incubated with anti-GPX1 and anti-PRRSV GP5 polyclonal antibodies (prepared and kept in our laboratory) and, subsequently, with HRP-conjugated goat anti-mouse IgG

(Sigma), followed by visualizing with diaminobenzidine (DAB) substrate (BOSTER, Wuhan, China).

2.5. Immunization schedule

Large-scale preparations of plasmid DNA, including pcDNA3.1, pcDNA3.1-SynORF5, and pcDNA3.1-GPX1-LSynORF5, were purified by Endofree Maxi Plasmid Kit (TIANGEN Biotech, Beijing, China), as per the manufacturer's instruction. The concentrations of the plasmids were measured by Ultraviolet spectrophotometer (Invitrogen, USA) and adjusted to a final concentration of 1 µg/µL with the elution buffer TB, respectively.

2.5.1. Experiment in mice

Six-week-old BALB/c mice were purchased from Yang Zhou University. The mice were randomly divided into three groups and acclimatized under controlled specific pathogen-free conditions for 1 week prior to the start of the experiment. Three groups of mice, seven mice per group, were immunized i.m. twice at 2-week intervals with 100 µg plasmid DNA of pcDNA3.1-GPX1-LSynORF5, pcDNA3.1-SynORF5 and the empty vector pcDNA3.1. Serum samples were collected at 2, 4 and 6 weeks after primary inoculation for serological tests. Six weeks after primary immunization, mice were sacrificed and splenocytes were isolated as described previously (Wang et al., 2007 and Xiao et al., 2004) for IFN- γ and lymphocyte proliferation assays.

2.5.2. Experiment in pigs

Twenty piglets weaned at 3 weeks of age were obtained from a PRRS-free farm in Nanjing. The piglets were confirmed negative for PRRSV by PCR and ELISA. The piglets were then randomly separated into four groups, housed in separate rooms at the animal facility of the Institute of Veterinary Medicine, Jiangsu Academy of Agricultural Sciences, Nanjing, Jiangsu Province. Two groups were vaccinated i.m. twice at 2-week intervals with 500 µg of pcDNA3.1-GPX1-LSynORF5 dissolved in 1 mL of PBS or pcDNA3.1-SynORF5 dissolved in 1 mL of PBS, respectively. The third group was injected i.m. once with commercial PRRSV attenuated vaccine (Highly Pathogenic Porcine Reproductive and Respiratory Syndrome Vaccine, Live Strain JXA1-R, Dahuanong, Guangdong, China). And the control group was immunized with 1 mL of PBS i.m.. Serum samples were collected from each pig at 14, 28, 35, 42 and 49 days post-immunization (dpi) to detect specific anti-PRRSV antibodies. At 28 dpi, fresh PBMCs were collected from inoculated pigs for lymphocyte proliferation, IFN- γ release assay.

All groups were challenged at 28 dpi with PRRSV strain JSKM i.m. (2 mL) with an infectious titer of 1×10^5 TCID₅₀/mL. The animals were monitored for an additional 21 days, and rectal temperatures and clinical signs were observed daily. Blood samples were collected at 0, 3, 5, 7, 10, 14 and 21 days post-challenge (dpc) for viraemia and antibody detection. At 14 dpc, fresh lymphocytes were separated from

the peripheral blood of piglets to detect specific cell-mediated immune (CMI) responses. At 21 dpc, all pigs were euthanized for pathological analysis.

2.6. Serological tests

Purified GP5 recombinant protein was used as coating antigen for the endpoint ELISA to determine the GP5-specific antibodies as described previously (Fang et al., 2006). The titers were expressed as the reciprocal of the highest dilution of sera producing ratio values of 2.1. Serum neutralization assays were performed with a modified method described by Ostrowski et al. (Ostrowski et al., 2002). Briefly, the collected sera samples were heat-inactivated for 30 min at 56°C, and serially diluted in twofold. Then the diluted samples were mixed with equal volume of HP-PRRSV strain JSKM containing 100 TCID₅₀ and incubated at 37°C for 1 h. The mixtures were added to Marc-145 monolayers in 96-well tissue culture plates, and incubated at 37°C with 5% CO₂, cells were examined daily up to 5 days for cytopathic effects (CPE). The neutralization titers were expressed as the reciprocal of the highest serum dilution resulting in complete neutralization. Each sample was run in triplicate.

2.7. Lymphocytes proliferation assay

Lymphocyte proliferation assay was performed using splenocytes from mice by lysis or peripheral blood mononuclear cells (PBMCs) of piglets by density centrifugation. The splenocytes or PBMCs were collected and stimulated with or without 20 µg/mL of PRRSV proteins, which were prepared by ultracentrifugating the PRRSV-infected

Marc-145 cells at $80,000 \times g$ for 2 h. Lymphocyte proliferation assays were performed as described previously (Xiao et al., 2004). T-cell stimulation was expressed as stimulation index (SI), which was calculated as the ratio of the average OD value of antigen-stimulated wells to that of un-stimulated wells.

2.8. IFN- γ release assay

The isolated lymphocytes (1×10^6 cells/mL) were cultured in 24-well plates at 37 °C in the presence of 5% CO₂, stimulated with 20 μ g/mL of PRRSV proteins. After 72 h incubation, the cells were centrifuged and the culture supernatant was harvested to examine the presence of IFN- γ with commercial mouse (Boster, Wuhan, China) or porcine (USCN, Wuhan, China) IFN- γ immunoassay ELISA kits following the manufacturer's protocol. The concentrations of IFN- γ in the samples were determined from the standard curves.

2.9. Real-time PCR analysis of IFN- γ mRNA expression

Lymphocytes or PBMCs were cultured in 24-well plates at 1×10^6 cells/mL for 18 h at 37 °C with 5% CO₂, with or without 20 μ g/mL of PRRSV proteins. Total RNA of the stimulated cells was extracted by Trizol (Invitrogen, USA) according to the manufacturer's instructions and quantified by Ultraviolet spectrophotometer. 1 μ g of RNA was reverse transcribed in a 20 μ L reaction mixture, with 4 μ L 5 $\times$ QRT Super Mix (Vazyme, Nanjing, China). The cDNA product (0.5 μ L) was amplified in a 25 μ L reaction mixture containing SYBR[®] Green Real-time PCR Master Mix (ToYoBo,

Shanghai, China), 0.2 μ M each of the forward and reverse gene-specific primers. Each RT-PCR was performed in triplicate. PCR amplifications were performed using an Applied Biosystems 7500 Real-Time PCR System (ABI). Thermal cycling conditions were 2 min at 50 °C, 10 min at 94 °C, and 40 cycles of 15 s at 94 °C and 1 min at 60 °C. Relative IFN- γ gene expression data were analyzed as described previously (Wang et al., 2007). The gene-specific primers used were the same as described previously (Li et al., 2009). For mouse-IFN- γ : TCAAGTGGCATAGATGTGGAAGAA/TGGCTCTGCAGGATTTTCATG. For mouse- β -actin: CACTGCCGCATCCTCTTCCTCCC/CAATAGTGATGACCTGGCCGT. For pig-IFN- γ : AGAATTGGAAAGAGGAGAGTGACAA/TGAATGGCCTGGTTATCTTTGA. For pig-GAPDH: ACATGGCCTCCAAGGAGTAAGA/GATCGAGTTGGGGCTGTGACT.

2.10. Analysis of CD4⁺ and CD8⁺ T-cells

At 28 dpi, 7 and 14 dpc, peripheral blood was collected from each pig. PBMCs were separated and transferred into a 1.5 mL centrifuge tube (1×10^6 cells). One milliliter of a fluorescence solution (100 mL 0.15 M PBS pH 7.4, 2% newborn bovine serum) was then added, and the tube was centrifuged at $170 \times g$ for 3–5 min. The supernatant was removed, and the pellet was resuspended in 300 μ L of cell fluorescence solution for staining with FITC mouse anti-porcine CD3 (SouthernBiotech, USA), PE mouse

anti-porcine CD8 (SouthernBiotech, USA) and SPRD mouse anti-porcine CD4 (SouthernBiotech, USA) fluorescent antibodies at 4 °C in the dark for 30 min. After washing twice with fluorescence solution and centrifugation at $170 \times g$ for 5 min, the supernatant was discarded. The cell pellet was resuspended in 500 μ L of fluorescence preservation solution (0.15 M PBS pH 7.4, 2% glucose, 1% formaldehyde, 0.1% NaN_3). Flow cytometry (FACS Canto II, BD, USA) was then used to count $\text{CD3}^+\text{CD4}^+$ and $\text{CD3}^+\text{CD8}^+$ T-cells in 10,000 cells, and percentages of $\text{CD3}^+\text{CD4}^+$ and $\text{CD3}^+\text{CD8}^+$ T-cells were determined. Data were analyzed using FlowJo version 7.6.1 software.

2.11. Quantification of PRRSV viral load by qRT-PCR

Total RNA in the serum samples were extracted by Trizol. Complementary cDNA was synthesized with the High Capacity cDNA Reverse Transcription Kit (TAKARA, Dalian, China) following the manufacturer's protocol. PRRSV was quantified using the standard curve method, established by our laboratory, and real-time PCR was performed to evaluate the PRRSV ORF7 mRNA level.

Real-time qRT-PCR amplification was carried out with Probe qRT-PCR supermix (TAKARA, Dalian, China) in a 20 μ L reaction mixture containing 10 μ L of $2 \times$ Supermix, 10 μ M of primer (F: 5'-TCAGCTGTGCCAAATGCTGG-3'; R: 5'-AAATGGGGCTTCTCCGGGTTTT-3'), 10 μ M of probe (5'-FAM-TCCCGGTCCCTTGCCTCTGGA-TAMRA-3'), 0.5 μ L of ROX Reference Dye, 9.5

μL of sterile distilled water and 1 μL extracted RNA. Reactions were run on an ABI 7500 detection system (Life Technologies, USA) following the manufacturer's instructions. For quantification, cDNA samples of known concentration of plasmid containing the PRRSV ORF7 gene were 10-fold serially diluted and used to generate the standard curve, ranging from 10^2 to 10^6 copies per reaction. The virus quantity of the unknown samples was determined by the cycle threshold (Ct) value and analysis of the dissociation product.

2.12. Histopathology

Lung samples of pigs were collected from the right cranial lobe and fixed in 10% neutral buffered formalin and 5-μm sections were cut and stained with hematoxylin and eosin as described previously (Jung et al., 2007).

2.13. Ethics approval

The immunization, challenge, collection of serum samples and separation of mice spleen cells and pigs PBMC were performed in strict accordance with the guidelines of Jiangsu Province Animal Regulations (Government Decree No. 45).

2.14. Statistical analysis

Student's t-test was used to compare the humoral and cellular immune responses, cytokine production between different groups. Differences were considered statistically significant when $P < 0.05$.

3. Results

3.1. Plasmid Construction and Verification

Single PCR product of around 648 bp and 676 bp of the GPX1 and LSynORF5 genes, respectively (Fig. 1), were amplified using the plasmids pcDNA3.1-GPX1 or pcDNA3.1-SynORF5, which were constructed and kept in our laboratory.

Plasmids of pMD¹⁹-T-GPX1 and pMD¹⁹-T-LSynORF5 were successfully constructed (Fig. 1C) and sequenced the nucleotide sequences of GPX1 and LSynORF5. The sequence results indicated that both the nucleotide sequences for GPX1 and LSynORF5 were 99% identical to published GPX1 (Acc no. NM_214201.1) sequences and the sequencing results of the pcDNA3.1-SynORF5. The predicted protein sequences for GPX1 were 100% identical to published GPX1 (Acc no. NP_999366.1) sequences, as determined by BLAST analysis. A similar result was observed from LSynORF5.

Restriction endonuclease double digestion was further proved that GPX1 and SynORF5 genes had been successfully recombined into pcDNA3.1 plasmid. The sizes of the digested fragments containing the inserted fragments were 648, 676, and 1324 bp, along with an estimated 5428 bp pcDNA3.1 vector band (Fig. 1D).

As shown in Fig. 2, two specific protein bands of 50 and 25 KDa, consistent with the expected molecular sizes of GPX1-LSynORF5 and SynORF5 genes, could be detected in the lysates of cells transfected with pcDNA3.1-GPX1-LSynORF5 or pcDNA3.1-SynORF5. There were no protein bands in the lysates of cells transfected with the empty vector. Therefore, the results showed that the inserted gene fragment GPX1 could be expressed and did not influence the in vitro expression of SynORF5.

3.2. Immune responses to recombinant DNA constructs in mice

3.2.1. Humoral immune response

Serum samples collected at 2, 4, and 6 weeks after the primary immunization were used to detect GP5-specific antibody by ELISA using purified GP5 protein as the antigen. As shown in Fig. 3A, at 4 weeks after primary immunization, the antibody titer in the group injected with pcDNA3.1-GPX1-LSynORF5 was significantly higher than the group inoculated with pcDNA3.1-SynORF5 ($P < 0.01$). And at 6 weeks after primary immunization, the difference of GP5-specific antibody titers was also significant between the groups immunized with pcDNA3.1-GPX1-LSynORF5 and pcDNA3.1-SynORF5 ($P < 0.05$).

The neutralization capacity of serum samples was also evaluated in vitro by using serum neutralization assays. As shown in Fig. 3B, even though there was no significant difference in PRRSV-specific neutralizing antibody titers between the groups of mice immunized with pcDNA3.1-GPX1-LSynORF5 and that of mice

receiving pcDNA3.1-SynORF5 at 2 weeks after primary immunization, a statistically significant difference could be observed at 4 and 6 weeks ($P < 0.05$). At 6 weeks post primary immunization, the PRRSV-specific neutralizing antibody titers were 1:14.86 and 1:8.57 for constructs containing and lacking the GPX1 gene, respectively. No detectable neutralizing antibodies ($<1:2$) were observed in sera from mice immunized with the empty vector during the experimental period.

3.2.2. Cellular immune responses

To measure the T-cell proliferative response, the splenocytes were collected from immunized mice at 6 weeks after primary immunization and the lymphocyte-proliferative assay was performed. As shown in Fig. 3C, pcDNA3.1-GPX1-LSynORF5 elicited higher lymphocyte proliferation than the construct pcDNA3.1-SynORF5 ($P < 0.05$), the stimulation indexes (SI) were 2.625 and 1.82, respectively. These results indicate that GPX1 can also enhance Th1-type immune response induced by DNA vaccine pcDNA3.1-SynORF5.

To profile the cytokine levels induced by the DNA plasmids, IFN- γ secretion in splenocytes restimulated with PRRSV protein was measured by ELISA. As depicted in Fig. 3D, the highest IFN- γ production of 404.5 pg/mL was induced in group immunized with pcDNA3.1-GPX1-LSynORF5, followed by the pcDNA3.1-SynORF5 and pcDNA3.1 inoculated groups. Quantitative real-time RT-PCR was also performed to analyze the level of Th1 cytokine IFN- γ mRNA expression in the

restimulated splenocytes. Consistent with the results of the IFN- γ ELISA assay, the mean relative IFN- γ mRNA expression from mice inoculated with pcDNA3.1-GPX1-LSynORF5 was 2.645-fold higher than that immunized with pcDNA3.1 and 1.542-fold higher than that received pcDNA3.1-SynORF5, (Fig. 3E).

3.3. Immune responses to recombinant DNA vaccine constructs in pigs

3.3.1 Humoral immune responses

The immunogenicity of the recombinant DNA constructs was further investigated in pigs. As shown in Fig. 4A, anti-PRRSV GP5-specific antibodies in pigs vaccinated with pcDNA3.1-GPX1-LSynORF5, pcDNA3.1-SynORF5 and commercial vaccine JXA1-R could be detected by ELISA at 14 dpi, and increased after the booster.

The neutralization capacity of sera from pigs was also investigated. As shown in Fig. 4B, PRRSV specific neutralizing antibodies were detected on the 14th day after primary immunization and elevated by the 28th day. Meanwhile, no neutralizing antibodies (< 1:2) against highly pathogenic PRRSV strain JSKM were detected in the PBS control group on the 14th and 28th days.

3.3.2. Lymphocyte proliferation and cytokine detection in pigs

At 28 and 42 dpi, PBMCs were isolated and restimulated in vitro with purified PRRSV (20 μ g/mL) to analyze cellular immune responses. The results showed that As shown in Fig. 4C, at 28 dpi, the construct pcDNA3.1-GPX1-LSynORF5 elicited

significantly enhanced lymphocyte proliferative responses ($P < 0.05$) compared with pcDNA3.1-SynORF5 ($P < 0.05$), at 28 dpi. Furthermore, similar results could be observed in the IFN- γ mRNA and IFN- γ expression assays at 28 dpi. However, at 28 and 42 dpi, there was no significant difference on lymphocyte proliferative responses between the groups immunized with the construct pcDNA3.1-GPX1-LSynORF5 and JXA1-R ($P > 0.05$) as well as IFN- γ mRNA and IFN- γ expression assays.

3.3.3. CD4⁺ and CD8⁺ T-cell subtype assays

Lymphocytes were isolated at 14 and 28 dpi and 7 and 14 dpc (35 and 42 dpi, respectively) and analyzed for CD3⁺CD4⁺ and CD3⁺CD8⁺T-cells by flow cytometry. As shown in Fig. 5, the percentages of CD3⁺CD4⁺ T-cells in the pcDNA3.1-GPX1-LSynORF5 and JXA1-R groups were significantly higher than the control group PBS at 14 dpi. After challenge, the number of CD3⁺CD4⁺ T-cells decreased at 35 dpi and slightly increased at 42 dpi in the pcDNA3.1-GPX1-LSynORF5 group, which was in opposition to the results observed in the group JXA1-R. By contrast, the number of CD3⁺CD8⁺ T-cells in the pcDNA3.1-GPX1-LSynORF5 and JXA1-R groups markedly increased at 35 dpi (7 dpc), but at 42 dpi, it was decreased to a similar level with that detected at 28 dpi. At 14, 28 and 42 dpi, there was no significant difference of the percentages of CD3⁺CD4⁺ and CD3⁺CD8⁺ T-cells between the groups of pcDNA3.1-GPX1-LSynORF5 and pcDNA3.1-SynORF5, but at 35 dpi, the percentage of CD3⁺CD8⁺ T-cells observed in the group pcDNA3.1-GPX1-LSynORF5 was significantly higher than the group pcDNA3.1-SynORF5 ($P < 0.05$).

416

417 3.4. Protective efficiency against PRRSV challenge

418 3.4.1. Clinical signs and body temperature change

419 After challenge with virulent PRRSV, all pigs in PBS control group had high fever
420 ($\geq 40^{\circ}\text{C}$) (Fig. 6). At 7 and 8 dpc, the temperature of the control group was as high as
421 41°C and displayed a range of clinical signs, including inappetence, lethargy, rough
422 hair coats, dyspnea, periocular edema, eyelid edema and light diarrhea. Though two of
423 the pigs in the pcDNA3.1-GPX1-LSynORF5 group had high fever during 10-13 dpc,
424 the mean level of temperature at each point was lower than the group immunized
425 pcDNA3.1-SynORF5, and obvious clinical signs were not observed in the other pigs
426 in this group. No obvious clinical sign were also not observed in the JXA1-R group.

427

428 3.4.2. Viraemia after challenge

429 Blood samples of the pigs were collected at 0, 3, 5, 7, 10, 14, and 21 dpc, and
430 viraemia was monitored by real-time PCR. During the 21 days after challenge with
431 JSKM, pigs inoculated with pcDNA3.1-GPX1-LSynORF5 showed a lower level of
432 viraemia in blood than that of the control group ($P < 0.05$) (Fig. 7). At 5, 14 and 21
433 dpc, pigs inoculated with pcDNA3.1-GPX1-LSynORF5 also showed a lower level of
434 viraemia in blood than that of the group immunized pcDNA3.1-SynORF5 ($P < 0.05$).
435 However, there was a large difference in virus level between the pcDNA3.1-GPX1-
436 LSynORF5 and JXA1-R-treated groups, with the plasmid-treated animals showing
437 higher levels of viraemia than those treated with the commercial vaccine. This

difference was likely caused by the relatively poor humoral immune response induced by pcDNA3.1-GPX1-LSynORF5.

3.4.3. Pathological examination

At 21 dpc, the lungs were examined for gross lesions in all the lobes. On histological examination, lungs lesions in the control pigs were characterized by thickened alveolar walls, extensive infiltration with macrophages, and increased amounts of bronchiole exudate. Interstitial pneumonitis could be observed in pcDNA3.1-SynORF5 group. However, this interstitial pneumonitis was largely absent in pcDNA3.1-GPX1-LSynORF5 and JXA1-R groups (Fig. 8)

4. Discussion

PRRS is a viral disease causing catastrophic economic losses in pig industry worldwide. Even though killed and attenuated live vaccines are commercially available, they provide only partial protection and limited efficacy against heterologous challenge. It has been reported that GPX1 belongs to the family of selenoproteins, which can regulate the function of the immune system through humoral, cellular and nonspecific immunity. Furthermore, it was demonstrated that selenoproteins may be considered as a vaccine adjuvant in triggering robust immune response against breast cancer (Yazdi et al., 2015).

In this study, the pcDNA3.1-GPX1-LSynORF5 plasmid was successfully constructed, and co-expressed porcine GPX1 and the GP5 encoding-gene of PRRSV, with a GPGP flexible linker inserted between the two genes. This was done to minimize the conformational changes in tertiary structure and provide flexibility of the peptide chain. The constructed pcDNA3.1-GPX1-LSynORF5 plasmid was inoculated into mice and pigs to demonstrate that whether GPX1 could act as a molecular adjuvant, thereby enhancing the immune response induced by pcDNA3.1-SynORF5.

First, in our previous studies, we demonstrated that genetically modified GP5 could increase vaccine immunogenicity, but could not provide complete protection against PRRSV challenge (Li et al., 2009). Second, new vaccines are most frequently compared to PRRSV modified live vaccines, so in this study, we chose to evaluate only the vaccine plasmid incorporating GPX1 as adjuvant, compared with a commercially available attenuated virus vaccine in pigs.

Even though the mechanisms of adaptive immunity that play important roles in mediating vaccine-induced protection have not been completely elucidated, it is broadly accepted that neutralizing antibodies may represent a valuable parameter by which to evaluate the efficacy of a vaccine against PRRSV (Lopez et al., 2007 and Lopez and Osorio, 2004). Furthermore, cell-mediated immunity, particularly the level of virus-specific IFN- γ , has been described as another potential correlate of protective immunity against PRRSV (Murtaugh et al., 2002 and Zuckermann et al., 2007). As shown in Fig. 3, pcDNA3.1-GPX1-LSynORF5 induced significantly enhanced

humoral and cellular immune responses in mice compared with animals immunized with pcDNA3.1-SynORF5. We also found that the cellular immune response induced by pcDNA3.1-GPX1-LSynORF5 in pigs was comparable to that induced by JXA1-R vaccination, even though the humoral immune response induced by the plasmid vaccine was not significantly enhanced compared with JXA1-R. In evaluating these cell-mediated immune response to the vaccines, the frequencies of CD3⁺CD4⁺ and CD3⁺CD8⁺ T-cell subgroups in the pcDNA3.1-GPX1-LSynORF5 vaccinated group were found to be significantly higher than those from the commercial PRRSV vaccine group ($P < 0.05$) at 28 and 35 dpi. Furthermore, the T-cell subgroups in the pcDNA3.1-GPX1-LSynORF5 vaccinated group peaked simultaneously, with the numbers of CD3⁺CD4⁺ and CD3⁺CD8⁺ T-cells reaching maximal levels at 35dpi. The results demonstrated that the combination of GPX1 and PRRSV ORF5 could enhance CD3⁺CD4⁺ and CD3⁺CD8⁺ T-cell proliferation. At 14 dpc, the percentages of CD3⁺CD4⁺ and CD3⁺CD8⁺ T-cells were decreased in the pcDNA3.1-GPX1-LSynORF5 and JXA1-R vaccinated groups, but the frequencies of CD3⁺CD4⁺ and CD3⁺CD8⁺ T-cells in JXA1-R vaccinated groups were higher than the group vaccinated with the construct pcDNA3.1-GPX1-LSynORF5. These results could explain the more severe clinical signs observed in the plasmid-treated group compared with the JXA1-R vaccinated animals, because lower levels of CD4⁺ T-cells increases the likelihood of severe infection (Ren et al., 2014).

Selenium status may affect the function of cells of both adaptive and innate immunity. In the form of selenoproteins, Se plays an essential regulatory role in phagocytes by maintaining a proper balance between the positive and negative effects of reactive oxygen species (ROS). Thus, insufficient levels of selenoprotein such as GPXs, TRs and other that regulate ROS and the redox state of these cells may cause oxidation-induced death of phagocytes (Hoffmann, 2007). Furthermore, sufficient levels of GPX1 are necessary for regulating H₂O₂, which is needed in appropriate levels for the activation of phagocytes. It was demonstrated that endogenously produced ROS in dendritic cells and T cells played an important role in antigen specific communication and thus the initiation of adaptive immunity (Matsue et al., 2003). Supranutritional selenium promotes proliferation and favors differentiation of naive CD4⁺ T lymphocytes toward T helper 1 phenotype, thus supporting an acute cellular immune response, whereas excessive activation of the immune system and ensuing host tissue damage are counteracted through directing macrophages toward the M2 phenotype (Steinbrenner et al., 2015). Thus, it is perhaps not unexpected that a more significantly enhanced cellular immune response was observed compared with the humoral immunity induced by pcDNA3.1-GPX1-LSynORF5. Through analyzing the results from these animal experiments, GPX1 may prove to be a useful molecular adjuvant in improving PRRSV immune response to vaccination.

5. Conclusions

We successfully constructed a recombinant plasmid pcDNA3.1-GPX1-LSynORF5 in this study. Our results confirmed that the recombinant plasmid could induce stronger cellular and humoral immune responses than pcDNA3.1-SynORF5 in mice and pigs. Moreover, the recombinant plasmid could provide better protection against PRRSV challenge than pcDNA3.1-SynORF5 in pigs. Furthermore, similar cellular immune responses were observed between pigs immunized with pcDNA3.1-GPX1-LSynORF5 and attenuated commercial vaccine JXA1-R. Therefore, GPX1 may be a promising candidate molecular adjuvant for use in development of more effective vaccines.

Acknowledgments

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694

695

Table 1

Primer sequences for amplification of porcine GPX1 and LSynORF5.

Name	Sequence	Amplified gene
GPX1-F	GAGGGTACCATGTGCGCCGCTCAGCGTTCC <i>Kpn</i> I	GPX1
GPX1-R	GCCGAATTCTGGGAAAGGGGAAGCTGGGAT <i>EcoR</i> I	
LSynORF5-F	GCCGAATTCGGGCCCCGGACCTACCATGTTGGG <i>EcoR</i> I	LSynORF5
LSynORF5-R	GAAAGTGCTT GCACTCGAGCTAGAGACGACCCCATAGTTC <i>Xho</i> I	

Figure legends

Fig.1. (A) Subcloning of the GPX1 gene. Lane 1: Marker DL2000; lane 2: PCR products of GPX1. (B) Subcloning of the LSynORF5 gene. Lane 1: Marker DL2000; lane 2: PCR products of LSynORF5. (C) pMD¹⁹-T-GPX1 and pMD¹⁹-T-LSynORF5 digested with *Kpn* I /*Eco*R I and *Eco*R I /*Xho* I , respectively. Lane 1: Marker DL2000; lane 2: pMD19-T-LSynORF5 digested with *Eco*R I /*Xho* I ; lane 3: pMD¹⁹-T-GPX1 digested with *Kpn* I /*Eco*R I . (D) pcDNA3.1-GPX1-LSynORF5 digested with *Kpn* I /*Eco*R I , *Eco*R I /*Xho* I and *Kpn* I /*Xho* I , respectively. Lane 1: Marker DL2000; lane 2: pcDNA3.1-GPX1-LSynORF5 digested with *Kpn* I /*Eco*R I ; lane 3: pcDNA3.1-GPX1-LSynORF5 digested with *Eco*R I /*Xho* I ; lane 4: pcDNA3.1-GPX1-LSynORF5 digested with *Kpn* I /*Xho* I .

Fig. 2. Expression of the protein encoded by the recombinant plasmid pcDNA3.1-GPX1-LSynORF5 in the transfected cells. Approximately 70-80 % confluent vero cells were transfected with 2 µg of pcDNA3.1-GPX1-LSynORF5 (lane 3), pcDNA3.1-SynORF5 (lane 1) and control vector pcDNA3.1 (+) (lane 4), respectively. At 48 h post-transfection, the cells were collected and subjected to Western blot as described in Section 2. Protein standards are the lane 2.

Fig 3. The humoral and cellural immune response in mice induced by different DNA constructs. Serum samples were collected at 2, 4, 6 weeks after primary immunization to determine GP5-specific ELISA antibody (A) and neutralizing antibody (B) titers. Mouse splenocyte samples (n=7) were collected at 6 weeks after primary immunization and restimulated *in vitro* with purified PRRSV proteins (20 µg/mL). Lymphocyte proliferation assay (C), concentration (pg/mL) of the Th1-type cytokine IFN-γ (D), and the level of IFN-γ mRNA expression in immunized mice (E) were measured using the relevant references. Data represent the mean and S.E.M. for 7 mice per group.

Fig 4. Detection of humoral and cellural immune response in pigs induced by PBS, pcDNA3.1-SynORF5, pcDNA3.1-GPX1-LSynORF5 and JXA1-R, respectively. The immune protocol was described in section 2. Serum samples were collected at 14, 28, 35, 42 and 49 dpi to determine the GP5-specific ELISA antibody (A) and neutralizing antibody against JSKM strain (B). The PBMCs were separated at 28, 35 and 42 dpi and restimulated *in vitro* with purified PRRSV proteins (20 µg/mL). Lymphocyte proliferative assay (C), IFN-γ ELISA (D), and IFN-γ quantitative RT-PCR (E) were performed in Section 2. Data are presented as the mean ± S.E.M.

Fig 5. Flow cytometric analysis of proportions of CD3⁺CD4⁺ (A) and CD3⁺CD8⁺ (B) T-cell subpopulations in fresh PBMCs isolated from different groups of pigs. at 28, 35, and 42 dpi. Results were obtained by averaging data from the five serum samples (n=5) of each group. Data are shown as mean $\pm$ S.E.M.

Fig 6. Rectal temperature of pigs in each group. Rectal temperature of each pig was daily measured after PRRSV JSKM strain challenge. Data are shown as mean $\pm$ S.E.M.

Fig 7. Viraemia of pigs inoculated with different vaccine formulations followed by challenge with the PRRSV JSKM strain. Viral RNA of serum from each pig in different groups (n=5) was detected.

Fig.8. Examination of histological lesions in lungs of pigs in the PBS (A), pcDNA3.1-SynORF5 (B), pcDNA3.1-GPX1-LSynORF5 (C) and JXA1-R (D) at 21 dpc.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8