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Construction and immunogenicity of a recombinant pseudorabies virus co-expressing porcine circovirus type 2 capsid protein and interleukin 18

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ABSTRACT

A novel recombinant pseudorabies virus (PRV) expressing porcine circovirus type 2 (PCV2) capsid protein and IL-18 was constructed. The PCV2 open reading frame 2 (ORF2) and porcine IL-18 genes were amplified by PCR and then inserted into the PRV transfer vector (pG) to produce a recombinant plasmid (pGO18). Plasmid pGO18 was transfected into porcine kidney cell (PK15) pre-infected with PRV HB98 vaccine strain to generate a recombinant virus. The recombinant virus PRV-ORF2-IL18 was purified by green fluorescent plaque purification and the inserts were confirmed by PCR, enzyme digestion, sequencing, and Western blot. The humoral and cellular responses induced by the recombinant virus were assessed in mice. Mice ($n = 10$) were immunized with PRV-ORF2-IL18, PRV-ORF2, PRV HB98, or inactivated PCV2. PRV-ORF2-IL18 elicited high titers of ELISA and serum neutralizing antibodies and strong cell-mediated immune responses in mice as indicated by anti-PCV2 ELISA, PRV-neutralizing assay, PCV2 specific lymphocyte proliferation assay, $CD3^+$, $CD4^+$ and $CD8^+$ T lymphocytes analysis, respectively. And PRV-ORF2-IL18 immunization protected mice against a lethal challenge of a virulent PRV Fa strain and significantly reduced the amount of PCV2 viremia. These results suggest an adjuvant effect of IL-18 on cellular immune responses. The recombinant virus might be an attractive candidate vaccine for preventing PCV2 and PRV infections in pigs.

1. Introduction

Porcine circovirus type 2 (PCV2) belongs to the *Circoviridae* family of viruses and has a single-stranded circular DNA genome. In the late 1990s, PCV2 was identified as a causative agent of post-weaning multisystemic wasting syndrome (PMWS). The clinical symptoms of PMWS in nursery and feeder pigs include progressive weight loss, respiratory signs, and jaundice. Histologically, PMWS is characterized by lymphocyte depletion in the lymphoid follicles, and an influx of macrophages (Allan et al., 1999; Meehan et al., 1998). The mortality rate of PMWS has been reported to be 5–30% in affected pigs (Zhai et al., 2014).

PCV2 has two major open reading frames (ORFs), ORF1 and ORF2. ORF1 encodes Rep and Rep′, two proteins that are essential for viral DNA replication (Mankertz et al., 2004). ORF2 encodes the 28 kDa PCV2 capsid protein that self-assembles into virus-like particles with a morphology similar to PCV2 virions (Kim et al., 2002; Nawagitgul et al., 2000). Neutralizing monoclonal antibodies to PCV2 recognize the capsid protein (McNeilly et al., 2001), making the capsid protein an attractive candidate for developing a recombinant PCV2 vaccine (Blanchard et al., 2003).

Porcine pseudorabies virus (PRV) is a member of family *Herpesviridae*, genus *Varicellovirus*, subfamily *Alphaherpesvirinae* that causes respiratory problems, nervous symptoms, and abortions in affected pigs, and PRV has serious economic consequences (Nauwynck et al., 2007). PRV has a linear DNA genome of approximately 150 kb. It contains several envelope glycoproteins that interact with host cells. Foreign genes can be stably inserted and expressed in the PRV genome, making attenuated PRV strain a promising live vector for multivalent vaccines against

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pseudorabies and other swine diseases. The gG glycoprotein is an early viral protein expressed from one of the strongest PRV promoters. The gG promoter has been commonly used to drive the expression of foreign proteins (Qian et al., 2004; Thomsen et al., 1987).

Interleukin 18 (IL-18) was first described as an IFN-γ inducing factor. It is now recognized as a multifunctional cytokine that augments both innate and acquired immunity, and potentiates Th1 and Th2 immune responses (Gracie et al., 2003). Mature IL-18 is an 18 kDa secreted glycoprotein that is produced from pro-IL-18, a 23 kDa protein that is cleaved by caspase-1 and proteinase-3 (Sugawara et al., 2001). Activated macrophages are the main source of IL-18, although it is also constitutively expressed by corneal epithelial cells, keratinocytes, renal tubular epithelial cells (Burbach et al., 2001), and the airway epithelium during Th1 and Th2 immune responses (Faust et al., 2002).

Previous studies (Cheevers et al., 2001; Dong et al., 2013; Genmei et al., 2011; Wang et al., 2013; Zhang et al., 2013) have shown that vaccines containing a cytokine(s) and a protective antigen protein can induce better immunity than the antigen alone. In this study, we constructed a novel recombinant PRV, lacking the gG protein, which co-expressed PCV2 capsid (Cap) protein and IL-18. We then assessed whether IL-18 was efficacious as an immune adjuvant that could enhance the protective immune response to recombinant PRV in mice.

2. Materials and methods

2.1. Viruses and cell lines

The PRV-Fa strain was purchased from the China Institute of Veterinary Drug Control, Beijing, China. The PRV HB98 vaccine strain was purchased from Wuhan Keqian Animal Biological Products Co., Ltd. The PCV2 HN strain was isolated from clinical specimens, and confirmed in the Key Laboratory for Animal-derived Food Safety of Henan Province, China. The viruses were propagated in the PK15 (porcine kidney) cell line purchased from the China Institute of Veterinary Drug Control, China, and the PK15 cell line was free of PCV-1. All cells were grown and maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS), 100 μg/mL of streptomycin, and 100 IU/mL of penicillin. The porcine IL-18 gene was amplified and confirmed in the Key Laboratory for Animal-derived Food Safety of Henan Province, China (Chen et al., 2009). The recombinant PRV-ORF2 virus was constructed in the Key Laboratory for Animal-derived Food Safety of Henan Province, China (Chao et al., 2014).

2.2. Primers

The gG nucleotide sequences from the PRV-Fa strain, the SV40 polyA nucleotide sequences from the pcDNA3.1(+) vector (Invitrogen, USA), the EGFP nucleotide sequences from the pEGFP-N1 vector (Clontech, Palo Alto, CA, USA), the ORF2 nucleotide sequences of PCV2, and the IL-18 gene were retrieved from GenBank and aligned using the software program Lasergene (DNASTAR, Inc., Madison). The primers were designed using Primer Select (version 2.0) and were based on highly conserved sequences within the gG region of PRV, SV40 polyA region of the pcDNA3.1, EGFP region of the pEGFP-N1, ORF2 region of PCV2 and the full-length porcine IL-18 gene (Table 1). All primers were synthesized by Takara, China.

2.3. Construction of the recombinant plasmids

To construct the recombinant plasmid pGO18 containing the ORF2 and IL-18 genes, a PRV transfer vector, termed pG, was constructed first. To make pG, a 3100 bp fragment from PRV-Fa containing partial genes for the pK and gD proteins, and a complete gG gene, was amplified using the primers in Table 1. The product was then cloned into the pUC19 vector, between restriction sites for *sph*I and *Kpn*I to create the recombinant plasmid pUP. Then, pUP and a 300 bp fragment of the pcDNA3.1(+) SV40 polyA sequence that was amplified from the pcDNA3.1(+) vector were digested with *Bam*HI and *Pst*I. The 300 bp fragment was cloned into pUP to create pUPS. Then a 1660 bp fragment was amplified from the pEGFP-N1 vector using the primers in Table 1, and excised by *Pst*I digestion. The pEGFP-N1 fragment was then cloned into pUPS after it had been restricted with *Hin*dIII and blunted with the klenow fragment of DNA polymerase, and then restricted with *Pst*I. pG was the final intermediate plasmid containing the 3100 bp fragment from PRV-Fa, the 300 bp SV40 polyA sequence, and pEGFP-N1 fragment (Fig. 1).

Next, a 702 bp fragment of the ORF2 gene was amplified from the PCV2 HN strain. The ORF2 PCR product was digested using *Bam*HI and inserted into the corresponding site of pG to produce a recombinant plasmid termed pGO. The IL-18 PCR product was digested using *Bgl*II, and cloned into similarly digested pGO, whose restriction site is located in the multiple cloning sites (MCS) of EGFP. The resulting recombinant plasmid, pGO18, contains 702 bp of the PCV2 HN ORF2 gene and 579 bp of the IL-18 gene. The ORF2 gene utilizes the gG promoter and poly (A) terminator from the pcDNA3.1(+) vector. The IL-18 gene utilizes the CMV promoter and SV40 poly (A) terminator of EGFP (Fig. 1). The correct recombinant plasmid pGO18 was confirmed by PCR amplification, restriction enzyme digestion, and sequencing.

Table 1
Primers designed for the construction of the recombinant PRV-ORF2-IL18.

Target gene	Primer sequence (5′–3′)[a]	Expected product (bp)
PRV(gG)	P1:ATGCTCGCACGCCGCAA P2: AAGGCAGAGCCCAATGAT	3100
pcDNA3.1(SV40)	P3:TTGGATCCATGCTGGAGTCATCGC P4: TTCTGCAGCAGGCTTCACACCTTATACT	300
EGFP	P5:TACTGCAGGATACCTGTGGATAACCGTA P6:CACTGCAGACAATTCACGCCTTAAGAT	1660
PCV2(ORF2)	P7:GGGGATCCATCACGCATCGATGG P8: GCGGATCCCATTCATTAAGGGTTA	702
IL-18	P9:GAAGATCTATCACGTAGCCAAGA P10: GCTCTAGACATTCATTAAGGGTTA	579

[a] Underlined nucleotides indicate restriction endonuclease cleavage sites used for cloning.

2.4. Generation and purification of recombinant virus PRV-ORF2-IL18

The PRV HB98 vaccine strain was inoculated onto monolayers of PK15 cells in 6-well plates and incubated at 37 °C for 2 h. Then the virus solution was aspirated. Cells in each well were washed three times with 3 mL of phosphate buffer saline (PBS, pH 7.4). Then, pGO18 was transfected into the PK15 cells infected with the PRV HB98 vaccine using Lipofectamine Reagent 2000 (Invitrogen, USA) according to the manufacturer's instructions. Twenty-four hours post-transfection, fluorescent EGFP was detected using a standard FITC filter-equipped fluorescent microscope (Nikon Eclipse TS100). When obvious cytopathic effects (CPE) were observed, the transfected cells containing the recombinant pseudorabies virus PRV-ORF2-IL18 were harvested, frozen and thawed for three times. The recombinant virus was selected by fluorescent plaque purification and confirmed as described below.