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Enhancement of immune response of piglets to PCV-2 vaccine by porcine IL-2 and fusion IL-4/6 gene entrapped in chitosan nanoparticles

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ABSTRACT

In order to develop a novel effective immunomodulator to enhance pig resistance against post-weaning multi systemic wasting syndrome (PMWS), a recombinant plasmid co-expressing pig interleukin-2 (IL-2) and fusion interleukin-4/6 (IL-4/6) genes, designated VRIL4/6-2, was constructed and encapsulated in chitosan (CS) nanoparticles prepared by the ionotropic gelation method. Then 21-day old piglets were divided into two groups and intramuscularly injected respectively with VPIL4/6-2-CS and saline along with the porcine circovirus-2 (PCV-2) vaccine. The blood was collected from each piglet on days 0, 7, 14, 28, 56 and 84 after vaccination to assay the immunological changes. Content of IgG2a, $CD4^+$, $CD8^+$ T cells increased significantly in the sera or blood of piglets treated with VPIL4/6-2-CS ($P < 0.05$). Furthermore, the expression level of IL-2, IL-4, IL-6, IL-15, TLR-2, TLR-7, Bcl-2, TNF-α, CD45 and STATs (STAT1, STAT2, STAT3, STAT4) genes were significantly elevated in the treated piglets respectively in different days after inoculation ($P < 0.05$). The growth weight gain of the treated piglets was markedly improved in comparison with the controls ($P < 0.05$). These indicate that VPIL-4/6-2 entrapped with chitosan nanoparticles is a safe and promising effective adjuvant to promote the immune response of pig to PCV-2 vaccination.

1. Introduction

Porcine circovirus type 2 (PCV-2) is a small nonenveloped virus with a single stranded DNA belonging to the family Circoviridae (Allan and Ellis, 2000; Gillespie et al., 2009). PCV-2 is the major causative pathogen of porcine circovirus-associated disease (PCVAD) which contains several syndromes, including post-weaning multi systemic wasting syndrome (PWMS), porcine dermatitis and nephropathy syndrome (PDNS), and porcine respiratory disease complex (PRDC) (Chae, 2005; Segalés, 2012; Segalés et al., 2005). PCV-2 infection is present in most pig-producing countries and causes serious economic loss to the pig industry due to popular mixed infection with other pathogens (Ge et al., 2012).

Nowadays vaccination is the most effective method to protect porcine from PCV2 and PCVD. Commercial vaccines are inactivated or attenuated PCV2 strain isolated in different places, and some engineering vaccines are based on the subunit PCV2a (Beach and Meng, 2012). These vaccines can significantly reduce clinical disease, viral load and mortality rate (Beach and Meng, 2012). While commercial vaccines could not prevent the spread or infection of PCV2 completely, nucleic acid vaccine and other vaccines based on PCV2b are still under development. Therefore, there are pressing need for novel effective adjuvant to improve the immunity of pig to commercial vaccines, and some molecules, like cap protein, flagellin, nanoscale emulsions and CD40L and GM-CSF has been tried to develop as immunoadjuvant for PCV2 vaccines (Bielinska and O'Konek, 2016; Huang and Liu, 2016; Sno and Cox, 2016; Zhu and Zhang, 2016; Li and Huang, 2016). However, the present available vaccines do not effectively prevent PCV-2 infection and fail to stop transmission (Beach and Meng, 2012). Therefore, it is crucial to raise the efficacy of PCV-2 vaccines against the immunosuppresive disease.

Previous studies have shown that cytokines can be promising

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adjuvants, due to their safety and potency, such as interferon gamma (IFN-γ) (Genmei et al., 2011; Wang et al., 2013), interleukin-2 (IL-2) (Kim et al., 2000; Yang et al., 2010), interleukin4 (IL-4) (Zhang et al., 2007), interleukin-6 (IL-6) (Li et al., 2011). IL-2, IL-4 and IL-6 are important cytokines which play key roles in normal cellular and humoral immune responses, produced by various animal cells. IL-2 has multiple biological functions, including boost T-cell proliferation and induction of T regulatory responses, augmentation of the cytotoxicity of T cells and NK cells, stimulation of proliferation of activated B lymphocytes, and induction of immunoglobulin secretion (Collins and Oldham, 1993; Lan et al., 2008). IL-4 can influence humoral and cellular immune responses, such as the production, class switching and secretion of immunoglobulin (Pasquini et al., 1997; Paul, 2015); IL-6 has been shown to promote interleukin gene expression, B cell differentiation, T cell activation, and to play an important role in acute phase responses (Kishimoto, 2010; Kopf et al., 1994).

We have previously demonstrated that pig IL-2 or IL-6 with CpG immunostimulatory sequences shuffled gene (Li et al., 2011; Yang et al., 2010), could effectively enhance animal resistance against pathogen infection. Moreover, the fusion protein of pig IL-4 and IL-6 can induce stronger proliferation of pig lymphoblasts than IL-4 and IL-6 individually (Yang et al., 2013; Zhang et al., 2007).

Therefore, based on our previous research, and in order to develop safe and cost-effective immunomodulators for pig PCV-2 vaccine, our experiment was designed to construct a co-expressed pig IL-2 and fusion IL-4/6 genes entrapped in chitosan (CS) nanoparticles, and to evaluate its effect on the growth and immune responses of piglets to PCV-2.

2. Materials and methods

2.1. Conventional vaccine

Inactivated PCV vaccine (Sichuan HuaPai Bio-pharmaceutical Co., Ltd., China) was used in this experiment as a conventional vaccine. It contains the most epidemic virulent PCV-2b virus strain in China, which was isolated and cloned in Zhejiang province, named as ZJ/C strain. One dose for pig vaccination includes the equivalent of $10^{8.0}$ $TCID_{50}$/ml of an inactivated strain of PCV-2b.

2.2. Recombinant plasmid construction

Plasmid VR1020 (Vical company, San Diego, CA, USA) was used as a eukaryotic expression vector. The cDNA for fused pig IL-4/IL6 gene and pig IL-2 gene were cloned respectively from recombinant plasmids containing fused pig IL-4/IL-6 and pig IL-2 genes stored previously in our lab, and named VRIL4/6 and VRIL2.

The 57-base pair 2A sequence 5′-GGA GAG GGC AGG GGA AGT CTT CTA ACA TGC GGG GAC GTG GAG GAA AAT CCC GGG CCA-3′ encoding amino acids GEGRGSLLTCGDVEENPGP, along with downstream tissue plasminogen activator (TPA) signal sequence, was used to generate a multicistronic cassette linking the fused pig IL-4/IL-6 and IL-2 genes to make a single fragment encoding the proteins. The linked fragment was inserted into plasmid VR1020 under control of the human cytomegalovirus (CMV) promoter using standard methods. The resulting plasmid was validated by sequencing and named VRIL4/6–2.

2.3. Large-scale preparation of plasmid DNA and detection of endotoxin

Escherichia coli (*E. coli*) DH5α cells were transfected with the recombinant plasmid and plated on Luria Bertani (LB) medium with 60 μg/ml kanamycin (Kana). Positive clones were screened after incubating in 37 °C and confirmed by PCR assay.

Positive clone with plasmid VRPIL4/6-2 was used to inoculate 400 ml LB broth with Kana at 60 μg/ml and shaken for 16 h at 37 °C. Bacterial cells were pelleted by centrifugation and plasmid DNA extracted following large-scale alkaline lysis. The plasmid was precipitated and purified by the spermine method (Murphy et al., 1999). Residual contamination by endogenous toxin of *E.coli* was < 0.1 EU/mg plasmid as measured by the Limulus amebocyte lysate test (Ogikubo et al., 2004). Plasmid DNA was resuspended in sterile water and stored at − 20 °C until use.

Table 1
The primers for QRT-PCR.

Gene	Oligonucleotide sequences (5′-3′)	Annealing temperature (°C)
PPIA-F	AGACAGCAGAAAACTTCCGTG	52.0
PPIA-R	ACTTGCCACCAGTGCCATTA	
CD45-F	GGACATGTGACCTGGAAACC	55.0
CD45-R	CCATTACGCTCTGCTTTTCC	
TNF-α-F	CCCACGTTGTAGCCAATGTC	60.0
TNF-α-R	GAGGTACAGCCCATCTGTCG	
IL-2-F	AGCTCTGGAGGGAGTGCTAA	60.0
IL-2-R	TGTTTCAGATCCCTTTAGTTCCA	
IL-4-F	GCTGCCCCAGAGAACACGAC	60.0
IL-4-R	AGGTTCCTGTCAAGTCCGCTC	
IL-6-F	ATAAGGGAAATGTCGAGGCTG	60.0
IL-6-R	GTGGCTTTGTCTGGATTCTTTC	
Bcl-2-F	GAAACCCCTAGTGCCATCAA	60.0
Bcl-2-R	GGGACGTCAGGTCACTGAAT	
IL-15-F	ACTGAGGATGGCATTCATGTC	57.5
IL-15-R	GCCAGGTTGCTTCTGTTTTAG	
TLR2-F	TGCTGCAAGGTCAACTCTCT	61.0
TLR2-R	CAGCAGGGTCACAAGACAGA	
TLR-7-F	ATAGCGAGCATCACTCCAGCC	60.0
TLR-7-R	TAATCTGCTGCCTTCTGGTGC	
STAT-1-F	TCTGGCACAGTGGCTAGAAAATC	56.3
STAT-1-R	GAAAACGGATGGTGGCAAAC	
STAT-2-F	AACATTCCTGAGAACCCACTG	54.0
STAT-2-R	CTGTTAGAGACCACGATGAGC	
STAT-3-F	AGGACATCAGCGGTAAGA	60
STAT-3-R	GGTAGACCAGCGGAGACA	
STAT-4-F	CCTGAAAACCCTCTGAAGTACC	53.7
STAT-4-R	CTGGGAGCTGTAGTGTTTACC	

F: forward. R: reverse.

Table 2
Pig weights during the 84 days of observation.

Group	Initial weight (Kg)	End weight (Kg)	Net gain (Kg)	Daily gain (Kg)
A	8.10 ± 0.98	45.50 ± 6.18	37.40 ± 5.76*	0.445*
B	7.35 ± 0.25	38.33 ± 4.50	30.98 ± 4.25	0.369

* Significantly different from controls ($P < 0.05$).

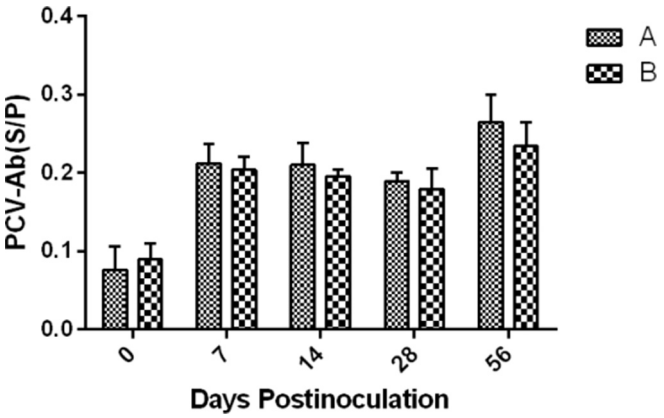

Fig. 1. Anti-PCV-2 antibody contents over the 56 days experimental period. Concurrently with a single IM dose of inactivated PCV-2 vaccine, group A received an IM injection of VPIL-4/6-2-CS; group B received saline. Note: Criteria standard: S/P = (S − N)/(P − N) = (sample OD - negative control OD)/(positive control OD -negative control OD), PCV-2 antibody was considered positive when the S/P ≥ 0.16.