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PED subunit vaccine based on COE domain replacement of flagellin domain D3 improved specific humoral and mucosal immunity in mice

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ABSTRACT

Porcine epidemic diarrhea (PED) is an important re-emergent infectious disease and inflicts huge economic losses to the swine industry worldwide. To meet the pressing need of developing a safe and cost-efficient PED maternal vaccine, we generated three PED subunit vaccine candidates, using recombined *Salmonella* flagellin (rSF) as a mucosal molecular adjuvant. Domain D3 in rSF was replaced with COE domain of PEDV to generate rSF-COE-3D. COE fused to the flanking C′/N′ terminal of rSF yielded rSF-COE-C and rSF-COE-N. As a result, rSF-COE-3D could significantly improve COE specific antibody production including serum IgG, serum IgA, mucosal IgA and PEDV neutralizing antibody. Furthermore, rSF-COE-3D elicited more $CD3^+CD8^+$ T cell and cytokine production of IFN-γ and IL-4 in mouse splenocytes. In summary, our data showed that rSF-COE-3D could improve specific humoral and mucosal immunity in mice, thus suggesting that rSF-COE-3D could be applied as a novel efficient maternal PED vaccine.

1. Introduction

PED, a highly contagious acute enteric diseases characterized by vomiting, watery diarrhea, dehydration and anorexia, inflicts huge economic losses to the swine industry worldwide [1,2]. The pathogen of the disease is characterized as PEDV, an enveloped RNA virus belonging to the genus Alphacoronavirus [3–5]. PEDV was first reported in European in 1971 [1]. Since late 2010, the PEDV variants caused continuous economic losses in pork industry of China [5–7] and attracted many concerns worldwide [8,9]. Control of this re-emergent infectious disease is of urgent need [1,2].

As PEDV causes high morbidity and mortality particularly in newborn piglets, vaccination aiming to improve the protective efficacy of lactating sow in order to protect its suckling neonates becomes the most available and efficacious protective measures against PED [10]. Many efforts have been made to develop effective vaccines, take modified-live vaccines (MLVs) and whole virus inactivated vaccines (WIVs) for instance [1,2,11]. However, because of the shedding problem of MLVs [2,12] and limited antigen dose of WIVs [1,2], development of safe and cost-efficient PED maternal vaccines is still a pressing need. Subunit vaccine, known to be safe and cost-efficient for targeted immunization, is a promising strategy for vaccine development [13–15].

Spike (S) protein, which is the most important targeting antigen, is composed of about 1386 amino acids (aa) and contains at least four neutralizing domains [4,16,17]. Particularly, the "collagenase equivalent" (COE) (residues 499-638) region, is a highly conservative neutralizing domain [4,17]. It could produce PEDV neutralizing antibodies in mice, when cooperated with molecular adjuvants [18–20]. Therefore, PED subunit vaccine based on the COE domain is expected to be a promising candidate.

Flagellin, a Toll like receptor (TLR) 5 ligand, was shown to potentiate the immunopotency and effectiveness of subunit vaccines as a molecular adjuvant [21–24]. Among the flagellin-adjuvanted vaccines, a quadrivalent seasonal flu vaccine VAX2012Q accomplished phase 1 safety and immunogenicity study [21–24]. The two conserved domains of *Salmonella* flagellin (SF) form a dimer with a TLR5 signaling motif in ND1 (89-96) as well as a Nod-like receptor protein 4 (NLCR4) signaling motif in CD0 (502-505), mediating the adjuvanticity of flagellin [25]. Flagellin provided good adjuvanticity for both C- and N-terminal fusion targeting antigen [26,27]. Although D0, D1 and D2 are proved to be responsible for flagellin's TLR5 agonist efficacy and adjuvanticity, antigen replacement of domain D3 in flagellin was reported to be a promising strategy for subunit vaccine design [28,29]. Furthermore, a suitable format of the flagellin-adjuvanted vaccine

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for different antigen was important on triggering TLR5 and enhancing the immunogenicity of the vaccine [30]. Thus, all of these three formats of flagellin-adjuvanted subunit vaccines were considered to be used for the development of novel effective PED maternal vaccines.

In this study, three PED subunit vaccine candidates, based on recombined SF (rSF) as a mucosal adjuvant, were generated. Domain D3 in rSF was replaced with COE domain of PEDV to generate rSF-COE-3D. COE domain was fused with C/N-terminal of rSF to generate rSF-COE-C and rSF-COE-N. Our data suggest that rSF-COE-3D, with the polymerizing ability and TLR5 agonist activity, could elicit significant systemic humoral, local mucosal and T cell immune responses in mice. Therefore, rSF-COE-3D can be expected to be a novel efficient maternal PED vaccine, thus providing a new insight into designation of vaccine for other enteric viral infections.

2. Material and methods

2.1. Cell lines, virus and vectors

Vero cells and Human epithelial colorectal adenocarcinoma (Caco2) cells from ATCC were cultured in DMEM supplemented with 10% FBS (Thermo Scientific), penicillin (100 units/mL), and streptomycin (100 mg/mL) at 37 °C with 5% CO_2.

The GDS01 PEDV strain (GenBank Accession No. 1070204) [5] was used for COE amplification.

FliC-L3A plasmid derived from pET-28a (+) vector (kindly provided by Prof. Huimin Yan) was used to express recombinant *Salmonella typhimurium* flagellin (rSF) protein as described previously [25].

Plasmid pBCX vector containing MsyB gene (kindly provided by Prof. Qingmei Xie) was used for prokaryotic expression.

2.2. Construction of plasmids

The COE fragments amplified from PEDV strain GDS01 (GenBank Accession No. 1070204) were introduced with flanking N′ and C′ terminal GGGSGGGS linkers. *EcoR* I-restricted rSFN (aa 2-220) fragments and *Xho* I-restricted rSFC (aa 320-506) fragments were amplified from FliC-L3A. rSF-COE-3D fragments was generated by overlapping of rSFN, COE and rSFC fragments in sequential order. The rSF (aa 2-506) fragment was amplified from FliC-L3A. COE fragments were combined to the flanking C′/N′terminal of rSF to generate rSF-COE-C and rSF-COE-N fragments. rSF-COE-3D, rSF-COE-C and rSF-COE-N fragments with *EcoR* I and *Xho* I cloning sites were inserted into pBCX vector to express rSF-COE-3D, rSF-COE-C and rSF-COE-N proteins respectively. COE fragments with N′ terminal *EcoR* I and C′ terminal *Xho* I restriction sites were inserted into pBCX vector to express COE protein. All primers used above were described in Supplementary Tables 1–3.

2.3. Expression and purification of recombinant proteins

Recombinant proteins were expressed and purified as previously described [31,32]. In brief, recombinant proteins were expressed in the E. coli BL21 (DE3), purified by affinity chromatography on a Ni-NTA column (GE, China) and dialyzed with 150 mM PBS. Contaminating lipopolysaccharide (LPS) was removed by Triton X-114 (Sigma, China) phase separation. The residual LPS content was determined to be less than 0.002 EU/μg using the Limulus assay (Xiamen Bio Endo Technology, China). Quantification of the purified proteins was performed using the Bradford assay. The purified protein was preserved in quantitative packing at −80 °C untill use.

2.4. Western blot analysis

Purified proteins were verified by western blotting with an anti-His-tag monoclonal antibody in 2000× dilution (Proteintech, China) and a secondary HRP-conjugated goat anti mouse IgG antibody (Southern Biotech, China) in 8000× dilution. Visualization of the antibody-antigen complex formation was accomplished using ELC (FDbio, China). Experiment was repeated at least three times.

2.5. Flagellin's polymerization assay

Purified rSF proteins were polymerized as previously described [29,33]. Briefly, A final buffer concentration of 1.5 M ammonium sulfate was obtained for each buffer + protein sample and samples were polymerized for 12–16 h at 25 °C. Polymers were pelleted with high-speed centrifugation at speed 100,000 ×*g* for 20 min at 4 °C and washed twice with 150 mM PBS. Polymer samples were then used for observation with negative stain by transmission electron microscope (TEM) (JEM1400, JEOL, Japan). Experiment was repeated at least three times.

2.6. Test of flagellin's TLR5 agonist efficacy

The flagellin TLR5 agonist efficacy was evaluated by testing their ability to inducing IL-8 in Caco-2 cells as previously described [32]. In brief, confluent monolayer Caco-2 cells were cultured overnight in medium without FBS, and the cells were stimulated with protein for 6 h at the indicated concentrations. The supernatants were then collected and quantified for IL-8 using Human IL-8 ELISA MAX™ Standard (Biolegend, USA) according to the manufacturer's instructions. Experiment was repeated at least three times.

2.7. Immunization and sample preparation in mice

90 six to eight-week-old female SPF BALB/c mice were randomly allocated into 6 groups and anesthetized immunized intranasally (i.n.) with 200 nM purified protein (10 μg for rSF, 9 μg for COE, 17 μg for rSF-COE-3D, 20 μg for rSF-COE-N or rSF-COE-N) or no protein in 10 μl PBS per dose for three times at two-week intervals.

Mice (n = 5 per group) were sacrificed and thoracolaparotomy was performed to harvest the splenocytes at 14 days after the first immunization. Immunized sera and mucosal secretions were collected 14 days later after the last immunization for analysis of PEDV-specific antibody responses as previously described [32]. Briefly, blood samples were collected from the retro-orbital plexus. Vaginal lavage fluid was obtained by washing the genital tract with 30 μl of sterile PBS three times. Thoracolaparotomy was performed after sacrifice to harvest the small intestines, which were then ground in 1.6 mL of grinding buffer (PBS with 0.2% saponin, 0.2% NaN_3 and 50 μM PMSF). The ground samples were then oscillated for 4 h at 4 °C with centrifugation. All samples were stored at −80 °C until they were assayed by ELISA. An amount of 100 μl of sera or mucosal samples was subsequently applied as a 3-fold dilution series for ELISA.

2.8. Flow cytometry analysis

For the detection of $CD4^+$ and $CD8^+$ T cell responses, splenocyte were obtained from vaccinated mice (n = 5) at 14 days after the first vaccination. Mouse lymphocyte separation medium (DKW33-R0100) (DKW, China) and nylon nets (DKW33-N25) (DKW, China) were used to separate splenocytes according to the manufacturer's instructions and the single-splenocyte suspensions were stained with 0.5% APC-labeled anti-CD3, 1% FITC-labeled anti-CD8a, 0.5% PE-labeled anti-CD4 and 5% 7-amino-actinomycin D (7-

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