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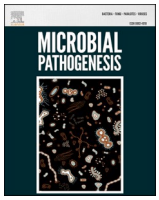

Development of a novel nanobody-fused flagellin adjuvant to enhance immunogenicity in a PCV2 subunit vaccine

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ABSTRACT

Porcine circovirus type 2 (PCV2) infection causes significant economic losses in the global swine industry. The capsid (Cap) protein serves as the core antigen in PCV2 subunit vaccines, but its weak immunogenicity necessitates adjuvants to enhance the induced immune responses. Bacterial flagellin proteins are ideal candidates for next-generation vaccine adjuvants. In this study, we designed a novel flagellin-based adjuvant by co-expressing bacterial flagellin with nanobodies (Nb) specific to the PCV2 Cap protein (Nbcap-flagellin). This recombinant fusion protein leverages the high-affinity binding ability of the nanobodies to bind the Cap protein, forming an antigen-adjuvant complex to enhance vaccine immunogenicity. We further evaluated the effect of this nanobody-fusion flagellin adjuvant to enhance the immune response in mice to a subunit vaccine with PCV2 Cap protein as the model antigen. The results demonstrated that vaccination of mice with PCV2-Cap and Nbcap-flagellin adjuvant (Cap + Nbcap-flagellin) vaccine induced significantly higher levels of ELISA titers of Cap-specific antibodies, neutralizing antibodies, and immune-related cytokines compared to the PCV2-Cap vaccine without adjuvant (Cap) and the mixture of Cap protein with flagellin (Cap + flagellin). Following virulent PCV2 challenge, addition of the Nbcap-flagellin adjuvant to the PCV2 subunit vaccine significantly reduced the viral load in the serum of PCV2-challenged mice and prevented pathological changes in lymphoid tissue. These findings suggested that the Nbcap-flagellin adjuvant could enhance the immune response and provide better protection against PCV2 infection, making it a promising candidate for improving the efficacy of PCV2 subunit vaccines.

1. Introduction

Porcine circovirus type 2 (PCV2), a non-enveloped virus with single-stranded circular DNA, belongs to the genus Circovirus within the family *Circoviridae* [1]. PCV2 exhibits high transmissibility among domestic and wild pigs, inducing multisystemic dysfunctions in respiratory, urinary, neurological, and reproductive systems [2]. It is considered to be a critical pathogen of porcine circovirus-associated diseases (PCVAD) such as postweaning multisystemic wasting syndrome (PMWS) and reproductive disorders, leading to substantial economic losses in the swine industry [3,4]. Current prevention strategies focus on routine disinfection, improved herd management, and vaccination across age groups, which effectively reduces viral transmission, mitigates tissue damage, and alleviates economic impacts [5]. Vaccination is the most effective preventive measure against PCV2. Subunit vaccines based on the capsid (Cap) protein, which can spontaneously self-assemble into antigenically intact virus-like particles (VLPs), are the preferred choice for vaccine development [6,7]. However, the limited immunogenicity of subunit vaccines may require additional antigen doses, powerful adjuvants [8], or in some cases, booster immunizations to achieve optimal protection. These limitations underscore the critical need for innovative strategies to enhance the immune-potency of PCV2 VLP-based vaccines.

Conventional adjuvants such as aluminum salts and Freund's adjuvant, while effective in enhancing immune responses, are associated with toxic side effects [9]. Flagellin, a conserved pathogen-associated molecular pattern and the primary protein component of bacterial flagella, has been reported as a potent mucosal vaccine adjuvant capable of eliciting dual protective immunity through toll-like receptor 5 (TLR5)

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mediated activation [10–12]. Its dual role as a virulence factor and immune stimulant has been harnessed in vaccine development and immunotherapy to enhance adaptive immune responses against co-administered antigens. Flagellin can function as an adjuvant either independently or through antigen fusion strategies, with the latter exhibiting better effect [13]. Research reported that the fusion of PCV2 Cap protein with Salmonella flagellin significantly improved immunogenicity and protective efficacy [12]. Nevertheless, the fixed antigen-to-adjuvant ratio inherent in such fusion recombinant proteins imposes limitations on further optimization of this critical parameter for maximal immune response. To address this issue, nanobodies (Nbs) emerge as a promising tool for optimizing antigen-adjuvant conjugation strategies in adjuvant development. Characterized by their small molecular size (15 kDa), strong antigen affinity, and remarkable structural stability, these single-domain antibodies exhibit superior tissue penetration compared to conventional antibodies [14,15]. Most importantly, the compact structure and chemical simplicity of Nbs enable precise conjugation between flagellin and Cap protein, allowing flexible adjustment of the antigen-adjuvant ratio while maintaining stable molecular interactions [16].

In this study, the recombinant fusion protein of anti-PCV2-Cap Nbs and flagellin (Nbcap-flagellin) was expressed in an *E. coli* expression system. We then investigated the potential effect of Nbcap-flagellin on enhancing the protective immune response to the Cap protein-based PCV2 subunit vaccine when co-administrated with the PCV2-Cap protein.

2. Materials and methods

2.1. Plasmids, cells, and viruses

Competent *E. coli* cells including DH5α and BL21 (DE3) were products of Solarbio Technology Co., Ltd. (Solarbio, Beijing, China). PCV2-Cap protein, expressed by prokaryotic expression system, was previously prepared and stored in our laboratory. The flagellin-linker gene fragment was synthesized by Sangon Biotech (Shanghai) Co., Ltd. and cloned into the pUC57 vector. The recombinant plasmid pUC57-flagellin-linker carries truncated form of the flagellin gene and (Gly4Ser) linker sequence. The recombinant plasmids, pCold-SUMO-Nbcap containing anti-PCV2-Cap Nbs gene and 6 × His-tag, was previously constructed and maintained in our laboratory [17].

The PK-15 (ATCC CCL-33) cell line was used for propagation of PCV2. PK-15 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10 % (v/v) heat inactivated fetal bovine serum (FBS; Gibco), penicillin (100 units/mL) and streptomycin (100 mg/mL) (Invitrogen, Carlsbad, CA, USA). PCV2 strain DBN-SX07 (GenBank accession: HM641752) was utilized for the challenge experiment.

2.2. Methods

2.2.1. Construction of recombinant plasmids

The flagellin-linker fragment was excised from the recombinant plasmid pUC57-flagellin-linker using *Sac*I/*Xba*I restriction enzymes (New England Biolabs, Ipswich, MA), gel-purified, and ligated into the *Sac*I/*Xba*I-digested pCold-SUMO vector to generate recombinant pCold-SUMO-flagellin. Subsequently, the Nbcap fragment was liberated from the pCold-SUMO-Nbcap plasmid via *Hin*dIII/*Nco*I digestion (NEB) and directionally cloned into the corresponding sites of pCold-SUMO-flagellin, yielding the final construct pCold-SUMO-Nbcap-flagellin. Ligation products were transformed into *E. coli* DH5α competent cells (Solarbio), followed by colony PCR verification. The flagellin-linker fragment and pET-32a (+) were digested with *Hin*d III/*Xho* I, and the flagellin fragment was then cloned into the linearized pET-32a (+) vector to generate the pET-32a-flagellin recombinant plasmid.

2.2.2. Preparation of recombinant fusion protein Nbcap-flagellin and recombinant protein flagellin

The correctly sequenced recombinant plasmids pCold-SUMO-Nbcap-flagellin and pET-32a-flagellin were transformed into *E. coli* BL21 (DE3) competent cells, respectively. Single colonies were selected and cultured overnight at 37 °C, 180 r/min in LB liquid medium containing 100 μg/mL ampicillin (Solarbio, Beijing, China). The overnight cultures were inoculated into fresh LB medium at a 1:100 ratio, followed by incubation at 37 °C with shaking at 180 rpm and induction with IPTG when they reached an optical density at 600 nm (OD600) of 0.6. Cells were collected after 8 h and lysed by sonication, and lysates were centrifuged to separate soluble/insoluble fractions. His-tagged proteins in supernatants were purified via Ni-NTA affinity columns (Changzhou Smart-Lifesciences Biotechnology Co., Ltd., Changzhou, China), with purity and identity confirmed by SDS-PAGE and Western blot. The recombinant proteins were confirmed by SDS-PAGE analysis and western blotting analysis using an anti-His-tag monoclonal antibody (Biodragon, Beijing, China). The purified recombinant proteins were quantitated by Protein Quantification Kit (BCA Assay) (Solarbio, Beijing, China) and endotoxins were removed using Triton-X114 (Solarbio, Beijing, China).

The binding activity of recombinant Nbcap-flagellin was evaluated by ELISA according to the method described in the literature with slight modifications [17]. Briefly, PCV2-Cap protein (10 μg/mL) was coated onto the wells of a 96-well enzyme-linked immunosorbent plate. The purified recombinant proteins were quantified and used as the primary antibody (diluted to 10 μg/mL) to react with the coated antigen. After washing, HRP-conjugated mouse anti-His antibody (Thermofisher MA1-80218, 1:1000) was added and incubated for 1 h. Following another wash, TMD solution (TransGen Biotech Co., Ltd., Beijing) was used for detection and absorbance was measured at 450 nm.

2.2.3. Immunization and challenge protection assay in mice

Experimental grouping and vaccine dose settings were performed as previously reported with slight modifications [18], as detailed in Table 1. Six-week-old female BALB/c mice were randomly divided into five groups (I, II, III, IV, and V), with 12 mice per group. Mice in group I, II, and III were administrated intramuscular injections of 10 μg of PCV2-Cap protein per mouse (Cap), 10 μg of PCV2-Cap protein combined with 4 μg of flagellin adjuvant per mouse (Cap + flagellin), and 10 μg of PCV2-Cap protein combined with 4 μg of Nbcap-flagellin adjuvant per mouse (Cap + Nbcap-flagellin), respectively. Groups IV and V were inoculated with 100 μL/mouse of PBS and served as control groups. The same immunization protocol was performed on Day 0 and Day 14, with a two-week interval between the two immunizations. Blood samples were collected from the retro-orbital venous plexus on days 7, 14, 21, and 28 post-primary immunization to assess ELISA antibody levels, neutralizing antibody titers, and the expression levels of IFN-γ and TNF-α in serum. Two weeks after the booster immunization, mice in groups I to IV were challenged with the PCV2-SDX07 strain ($10^{5.0}$ $TCID_{50}$/100 μL) via intraperitoneal injection under ether anesthesia. Group V received 100 μL of PBS as the negative control. Following the challenge, mouse body weight was monitored daily, and clinical symptoms, serum viral load,

Table 1
Overview of the mice immunization groups.

Group	Number of mice	Vaccine Immunization	Adjuvant	Challenge Strain
Group I	12	10 μg Cap	None	PCV2-SDX07
Group II	12	10 μg Cap	4 μg Flagellin	PCV2-SDX07
Group III	12	10 μg Cap	4 μg Nbcap-lagellin	PCV2-SDX07
Group IV	12	PBS	None	PCV2-SDX07
Group V	12	PBS	None	None

and histopathological changes were analyzed.

2.2.4. Indirect ELISA

PCV2-Cap specific antibody titers in serum were determined using an indirect ELISA with PCV2-Cap protein as the antigen. Microplates were coated overnight at 4 °C with PCV2-Cap protein, followed by washing with 0.05 % PBST (Tween-20 in PBS (pH 7.4), w/v). Plates were then blocked with 3 % MPBS (skimmed milk in PBS (pH 7.4), w/v) at 37 °C for 1 h, and serum samples were incubated at 37 °C for 1 h as primary antibodies. After additional washes, goat anti-mouse IgG1-HRP secondary antibody (1:5000) (Biodragon, Beijing, China) was added and incubated at 37 °C for 1 h. The reaction was developed with TMB substrate at 37 °C in the dark for 10 min and terminated with 2 mol/L H_2SO_4. Optical density was measured at 450 nm.

2.2.5. Serum neutralization assay

The micro-neutralization test was performed according to a previously published method [19], with some modifications. Serum samples were inactivated at 56 °C for 30 min, serially diluted twofold (1:2 to 1:256) in DMEM cell culture medium, and added to 96-well plates (triplicates per dilution). PCV2 virus was diluted to 200 $TCID_{50}$/mL in DMEM and mixed with serum dilutions (1:1 ratio) for 1 h at 37 °C under 5 % CO_2. The antibody-virus mixtures were then inoculated onto PK-15 cell monolayers (two wells per dilution, 100 μL/well) and incubated for 1 h. After removing the mixtures, cells were maintained in medium supplemented with 2 %–3 % serum for 48–72 h. The cells were fixed with 90 % acetone and then incubated with fluorescein isothiocyanate (FITC)-labeled goat anti-porcine IgG (Biodragon). The viral neutralization titers were calculated as the highest serum dilution showing more than 90 % neutralization of the well, as observed under a fluorescence microscope.

2.2.6. Detection of cellular immune responses

The potent adjuvant properties of flagellin have been attributed to the expression of TLR5 by many types of immune cells [20]. As the specific receptor for bacterial flagellin, TLR5 engagement with extracellular flagellin activates key transcription factors such as NF-κB, thereby inducing the production of multiple cytokines including TNF-α and IFN-γ in responsive cells [21,22]. To investigate the cellular immune factor levels in serum, serum samples were collected at 7 and 14 days postimmunization (dpi). The levels of IFN-γ and TNF-α in serum were quantified using the Mouse IFN-γ ELISA Kit and Mouse TNF-α ELISA Kit (Thermo Fisher), following the manufacturer's instructions. All samples were analyzed in triplicate, and cytokine concentrations were reported as pg/mL.

2.2.7. Real-time PCR analysis of PCV2 nucleic acid in serum

Blood samples were collected from all infected mice at 3 weeks post-challenge. Total nucleic acids were extracted using the TIANamp Virus DNA/RNA Kit (TIANGEN, Beijing, China) according to the manufacturer's protocol. PCV2 DNA was quantified by SYBR Green I-based quantitative real-time PCR (qPCR) using specific primers: PCV2-F (5′-ATAACCCAGCCCTTCTCCTACC-3′) and PCV2-R (5′-GGCCTACGTGGTCTACATTTCC-3′). Amplification conditions included an initial denaturation at 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s. Fluorescence signals were detected by the Light Cycler 480 System. The results were calculated as the mean of the logarithmic viral DNA copy number.

2.2.8. Histopathological assay

Lymph node tissues were harvested from euthanized mice at 3 weeks post-challenge. The tissues were fixed in 10 % neutral-buffered formalin at 4 °C for 24 h, then dehydrated, embedded in paraffin, and sectioned to a thickness of 4 μm. Hematoxylin and eosin (H&E) staining was performed, and histopathological changes were examined under a light microscope.

2.2.9. Statistical analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA). The statistical significance is indicated in the figures and figure legends. Error bars represent the standard deviation of the mean (SD), and $P < 0.05$ was considered statistically significant.

3. Results

3.1. Preparation of the recombinant Nbcap-flagellin fusion protein

3.1.1. Prokaryotic expression and purification of Nbcap-flagellin fusion protein

The recombinant plasmid pCold-SUMO-Nbcap-flagellin was transformed into *E. coli* BL21 (DE3). Under optimized induction conditions (1 mmol/L IPTG, 18 °C, 8 h), the Nbcap-flagellin fusion protein demonstrated predominant solubility with an apparent molecular weight of ~38 kDa as confirmed by SDS-PAGE (Fig. 1). Comparative analysis revealed maximal expression yields in induced cultures versus non-induced controls, with target protein predominantly localized in the soluble supernatant fraction post-lysis (Fig. 1A).

Following confirmation of recombinant protein expression, large-scale expression was performed, and the target protein was purified using a Ni^{2+} affinity chromatography column. The eluted protein samples were analyzed by SDS-PAGE. The results demonstrated that the Nbcap-flagellin fusion protein was successfully purified with high purity (Fig. 1B).

3.1.2. Analysis of the fusion protein

Western blot analysis was performed using a His-tag antibody to validate the expression of the Nbcap-flagellin fusion protein. A specific band was observed at approximately 38 kDa (Fig. 1C), confirming the successful expression of the recombinant protein.

To assess whether the fusion expression of Nbcap and flagellin fragments affects the binding activity of Nbcap-flagellin to the Cap protein, we determined the interactions using ELISA. The results demonstrated that Nbcap-flagellin retained high binding specificity to the Cap protein (Fig. 1F), suggesting that the fusion construct does not compromise its target recognition capability.

3.2. Expression and identification of flagellin recombinant protein

The pET-32a-flagellin plasmid was transformed into *E. coli* BL21 (DE3). Optimal protein expression was achieved by inducing the culture with 1 mmol/L IPTG at 30 °C and 160 rpm for 5 h. SDS-PAGE analysis was performed to determine the molecular weight and expression levels of the protein. The expressed flagellin protein had a molecular weight of approximately 25 kDa and was primarily present in the soluble fraction of the supernatant (Fig. 1D).

After confirming recombinant protein expression, large-scale production was carried out, and the flagellin recombinant protein was purified using a Ni^{2+} affinity chromatography column. The eluted protein samples were then analyzed by SDS-PAGE, and the results showed that the flagellin recombinant protein was successfully purified with high purity (Fig. 1E).

3.3. Immune-protective effect of the Nbcap-flagellin adjuvant

3.3.1. PCV2-specific humoral immune responses

The presence of Cap protein-specific antibodies in sera was detected using an ELISA assay with Cap protein-coated plates. Throughout the study, all mice administered PBS showed negative results for Cap protein antibodies. As shown in Fig. 2A, on Day 7, Cap protein antibodies were detected in all immunized groups except for the Cap protein group without an adjuvant. From Day 7 to Day 28, the antibody secretion levels in the groups I to III exhibited an increasing trend with successive immunizations. On Day 28, the PCV2-specific antibody level in mice

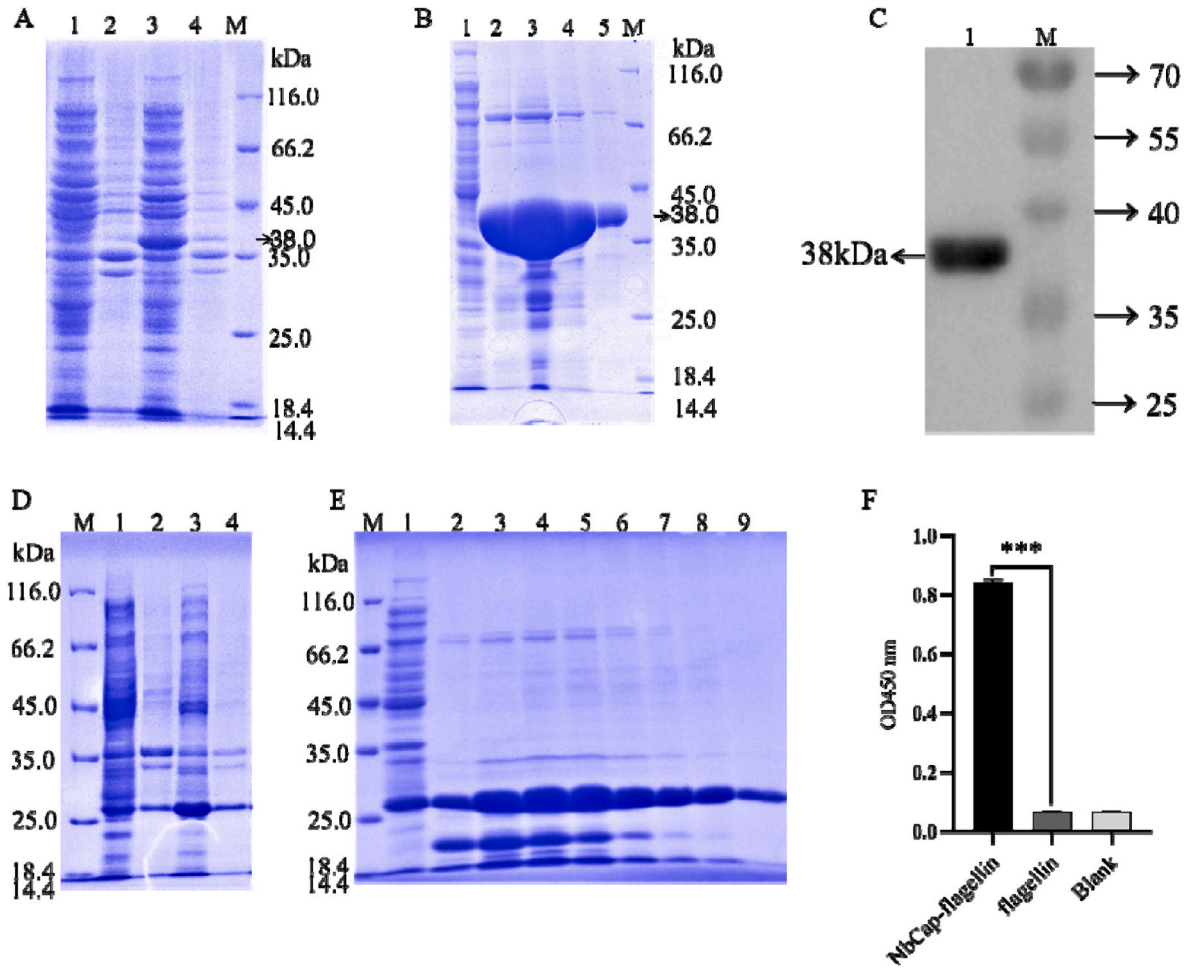

Fig. 1. Identification of the recombinant proteins. For bacterial protein expression samples, the culture volume was concentrated 10-fold using PBS. After sonicating the bacteria, the pellet was resuspended in an equal volume of PBS. The supernatant and the resuspended pellet were mixed with 5 × SDS loading buffer, and 10 μL of each sample was loaded into each well for SDS-PAGE analysis. For the purification samples, the elution fractions were mixed with 5 × SDS loading buffer, and 10 μL of each sample was loaded into each well for SDS-PAGE analysis. (A) Solubility analysis of expression of Nbcap-flagellin by SDS-PAGE. Lane M: protein molecular weight marker, lane 1: Supernatant of negative control bacteria after sonication, lane 2: Pellet of negative control bacteria after sonication, lane 3: Supernatant of recombinant bacteria after sonication, lane 4: Pellet of recombinant bacteria after sonication. (B) Purification analysis of Nbcap-flagellin by SDS-PAGE. Lane M: protein molecular weight marker, lane 1: Flow-through of Nbcap-flagellin recombinant protein, lanes 2 to 5: Purified Nbcap-flagellin recombinant protein. (C) Identification of Nbcap-flagellin by Western blot. Lane M: protein molecular weight marker, lane 1: Purified Nbcap-flagellin recombinant protein. (D) Solubility analysis of expression of flagellin by SDS-PAGE. Lane M: protein molecular weight marker, lane 1: Supernatant of negative control bacteria after sonication, lane 2: Pellet of negative control bacteria after sonication, lane 3: Supernatant of recombinant bacteria after sonication, lane 4: Pellet of recombinant bacteria after sonication. (E) Purification analysis of flagellin recombinant protein by SDS-PAGE. Lane M: protein molecular weight marker, lane 1: Supernatant of expression products, lanes 2 to 9: Purified flagellin recombinant protein. (F) ELISA confirmed the binding activity of Nbcap-flagellin to the Cap protein, with no interaction observed between the flagellin protein and the Cap protein.

vaccinated with the Nbcap-flagellin adjuvant vaccine was significantly higher than that in the flagellin adjuvant vaccine group and the non-adjuvanted vaccine group ($P < 0.05$).

The titer of PCV2 neutralizing antibodies were investigated. Throughout the study, all PBS-administered mice tested negative for PCV2 neutralizing antibodies. As shown in Fig. 2B, in mice immunized solely with the Cap protein vaccine, PCV2 neutralizing antibodies were detected in serum only on Day 21, with an antibody titer of 1:6.72. In the flagellin adjuvant vaccine group, neutralizing antibody titers became detectable on Day 14 (1:6.72) and continued to increase thereafter. In the Nbcap-flagellin recombinant fusion protein adjuvant group, neutralizing antibodies were detectable as early as Day 7. By Day 21, the neutralizing antibody titer was significantly higher than that in the non-adjuvanted vaccine group ($P < 0.05$). On Day 28, the titer reached 1:38, which was significantly higher than that in the flagellin adjuvant vaccine group ($P < 0.05$) and the non-adjuvanted vaccine group ($P < 0.01$).

These results indicated that, compared with the non-adjuvanted vaccine and the flagellin adjuvant vaccine, the Nbcap-flagellin adjuvant vaccine effectively induced higher levels of Cap protein antibodies and neutralizing antibodies in mice. The Nbcap-flagellin adjuvant significantly enhanced humoral immune responses in immunized mice.

3.3.2. *Analysis of cytokine levels in immunized mice*

Flagellin is a known agonist of TLR5 and has been demonstrated to upregulate the expression of certain cytokines, including TNF-α and IFN-γ, thereby enhancing cellular immunity [20]. To further determine the levels of TNF-α and IFN-γ induced by the Nbcap-flagellin adjuvant, the expression levels of these two cytokines were analyzed in mice sera.

As shown in Fig. 2C and D, throughout the study, the levels of TNF-α and IFN-γ in PBS-administered mice remained unchanged. From Day 7 to Day 28, the highest levels of TNF-α and IFN-γ secretion were observed in the Nbcap-flagellin adjuvant vaccine group compared with the flagellin adjuvant group and the non-adjuvanted vaccine group. On Day 28, the levels of TNF-α and IFN-γ in the Nbcap-flagellin adjuvant vaccine

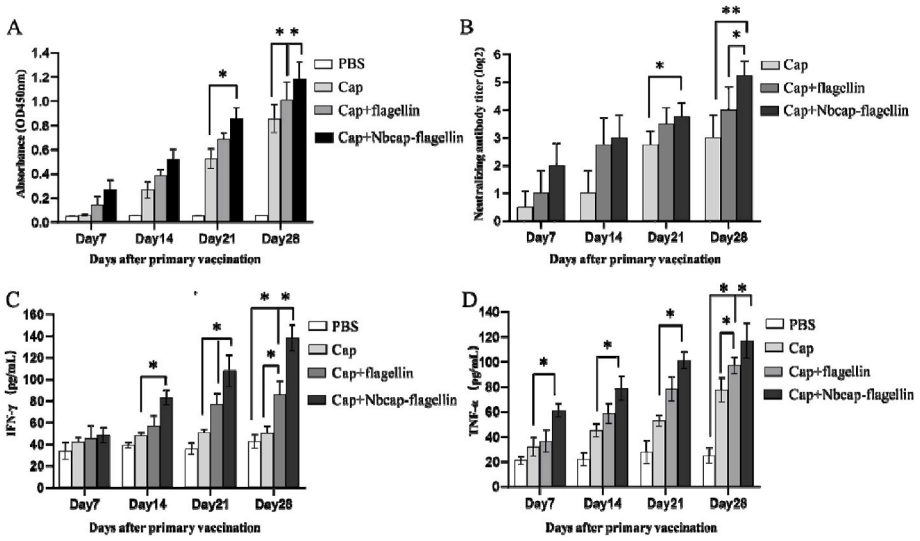

Fig. 2. Detection of PCV2-specific antibody, neutralizing antibody, and cytokines in sera at different times. (A) Serum antibody levels were analyzed by indirect ELISA using 100 ng/well of Cap protein as the coating antigen. (B) The viral neutralization titers PCV2 were detected by immunofluorescence assay and were determined as the highest serum dilution that exhibited >90 % neutralization of the well. The levels of IFN-γ (C) and TNF-α (D) in the serum samples were determined using ELISA kit. Data were shown as means ± S.D. Significantly differences are indicated by different superscripts (*) ($P < 0.05$).

group were significantly higher than those in the flagellin adjuvant group ($P < 0.05$). In addition, on Day 7, the TNF-α level in the Nbcap-flagellin adjuvant group was significantly higher than that in the non-adjuvanted vaccine group ($P < 0.05$). Only in the Nbcap-flagellin adjuvant group were the TNF-α and IFN-γ levels significantly higher than those in the negative control group on Day 28 ($P < 0.05$). These results indicate that the Nbcap-flagellin adjuvant induces higher levels of TNF-α and IFN-γ secretion in mice than the flagellin adjuvant. The Nbcap-flagellin recombinant fusion protein adjuvant effectively promotes a stronger cellular immune response.

3.3.3. *Quantification of serum viral load in mice following challenge*

The PCV2 DNA levels in serum samples collected three weeks after viral challenge were quantified using real-time fluorescence quantitative PCR method. In the unchallenged control group (Group V), no PCV2 viremia was detected (data not shown). As shown in Fig. 3F, all mice in the challenge control group (Group IV) exhibited PCV2 viremia, with an average viral genomic copy number of (5.0 ± 1.2) $\log_{10}$ copies/mL. Compared with the challenge control group, the non-adjuvanted vaccine group showed no significant reduction in PCV2 viremia ((4.2 ± 1.5) $\log_{10}$ copies/mL). Mice immunized with the recombinant fusion protein adjuvant vaccine exhibited a significantly lower level of viremia after PCV_2 challenge compared with other three challenged groups ($P <$

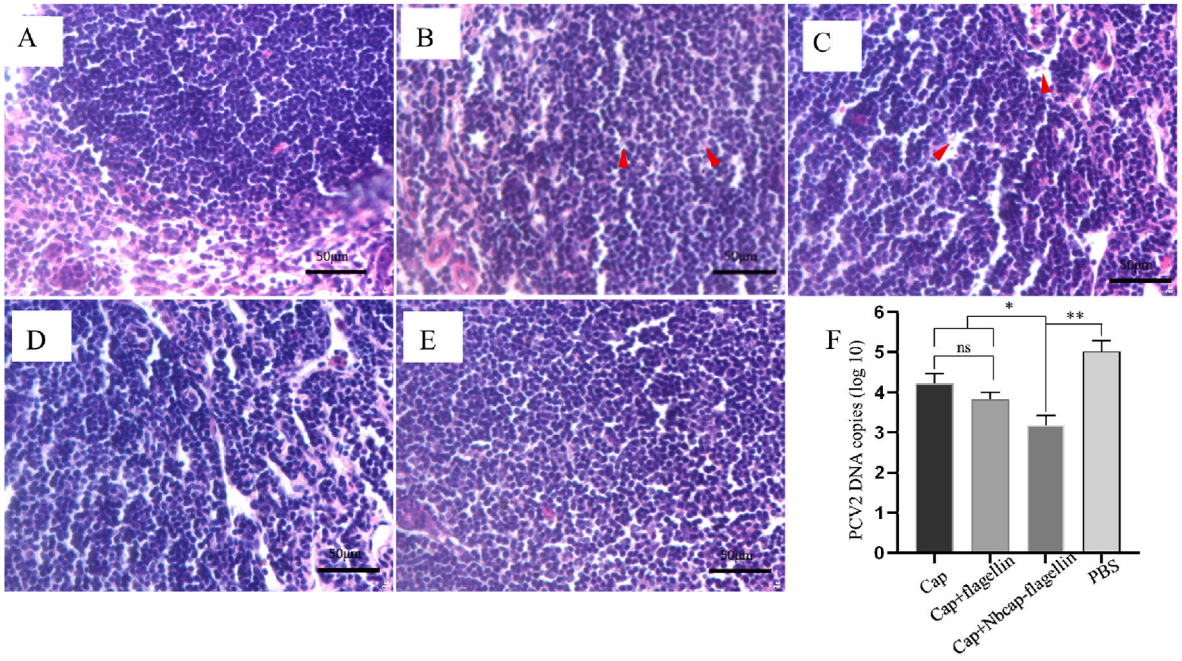

Fig. 3. Histopathological changes and serum viral load after PCV2 challenge in mice. (A–E) HE-stained lymph node sections (scale bar: 50 μm) at three weeks post-infection: (A) PBS control, (B) Challenge control (unvaccinated), (C) Cap protein vaccine, (D) Flagellin-adjuvanted vaccine, (E) NbCap-flagellin-adjuvanted vaccine. (F) Viral genomic copies in serum quantified by RT-PCR. Data expressed as log10 copies/mL. The serum viral load in Cap + Nbcap-flagellin group was significantly lower than other three groups ($P < 0.05$).

0.05), with an average genomic copy number of (3.1 ± 1.5) $\log_{10}$ copies/mL. The flagellin adjuvant vaccine group exhibited a lower serum viral load, but there was no significant difference compared to the non-adjuvant group. Among all groups, the Nbcap-flagellin adjuvant vaccine group exhibited the lowest PCV2 viral load.

3.3.4. Histopathological changes following viral challenge

Two weeks after the second vaccination, the mice were challenged with PCV2, and clinical symptoms as well as histopathological changes were observed. Three weeks post-challenge, inguinal lymph nodes were collected from the mice, and histological sections were prepared for pathological analysis.

No significant differences in body weight were observed among the groups following viral challenge, and no clinical symptoms or apparent pathological lesions were detected in any of the challenged mice. Histological examination revealed that the inguinal lymphoid tissues of the blank control group exhibited intact structural integrity, with no apparent tissue damage (Fig. 3A). As indicated by the arrows, the challenge control group showed lymphocyte depletion, lymphocyte apoptosis, inflammatory cell infiltration, and mild necrosis in the inguinal lymph nodes (Fig. 3B). In the non-adjuvanted vaccine group, a small number of necrotic lymphocytes were observed (Fig. 3C), while the flagellin adjuvant vaccine group exhibited mild infiltration of histiocytes and (Fig. 3D). The Nbcap-flagellin adjuvant vaccine group showed only minor tissue damage in the inguinal lymph nodes (Fig. 3E).

These results indicate that following PCV2 challenge, mice vaccinated with the Nbcap-flagellin adjuvant vaccine exhibited lower viremia and milder histopathological changes. The Nbcap-flagellin adjuvant vaccine provides enhanced immune protection in mice.

4. Discussion

PCV2 continues to pose a significant threat to global swine health due to its immunosuppressive properties and economic impact [23,24]. Immunization has been demonstrated to be a highly effective strategy for controlling PCVAD, as vaccination can significantly reduce the mortality rate caused by PCV2 infection. Compared with traditional inactivated vaccines, subunit vaccines offer enhanced safety. However, subunit vaccines generally require the addition of adjuvants to enhance their immunogenicity [25,26]. The incorporation of adjuvants can effectively reduce antigen dosage and the number of immunizations required, thereby minimizing the stress response in piglets associated with repeated vaccinations and improving immune protection [27]. Although traditional adjuvants exhibit strong immunostimulatory effects, repeated immunization may lead to neurotoxic effects [28,29]. To enhance vaccine efficacy, novel adjuvants are continually being developed. Studies have shown that flagellin stimulates innate immune responses through interaction with TLR5, thereby promoting both humoral and cell-mediated adaptive immunity, making it a promising novel immunological adjuvant [20,30,31]. In this study, a Nbcap-flagellin fusion protein was designed as an adjuvant, and its immunological efficacy was evaluated in a porcine circovirus subunit vaccine.

Nanobodies exhibit high affinity and strong tissue penetration. Compared with conventional antibodies, nanobodies offer advantages in terms of expression yield, solubility, and stability [32,33]. Due to their simple structure, nanobodies can be expressed in large quantities in prokaryotic expression systems, such as *E. coli* [34], or eukaryotic systems, such as yeast [35], through molecular cloning techniques. They have been widely applied in disease diagnosis and treatment. Studies have demonstrated that nanobodies can be directly used for therapeutic purposes. Tokuhara et al. [36] successfully expressed anti-rotavirus nanobodies in transgenic rice, and feeding the transgenic rice to mice effectively prevented rotavirus-induced diarrhea. Nanobodies have also been utilized as conjugates for targeted tumor therapy. Fang et al. [37] injected nanobody–anti-tumor drug conjugates into mice, effectively controlling tumor growth and metastasis. In this study, a flagellin-nanobody fusion protein was prepared using an *E. coli* system and employed as an adjuvant. The fusion protein was purified through nickel-affinity chromatography, yielding a highly soluble and stable protein with high purity. ELISA confirmed the binding activity of the Nbcap-flagellin recombinant protein to the Cap protein. We hypothesized that the Nbcap-flagellin fusion protein formed a complex with Cap, enabling the targeted co-delivery and co-processing of both the Cap and flagellin components within the same antigen-presenting cell (APC). The Nbcap-flagellin fusion protein adjuvant significantly enhanced the immune response elicited by the subunit vaccine.

Previous studies have demonstrated that fusion expression of PCV2 Cap protein with Salmonella flagellin exerts potent immunostimulatory and protective effects [12]. However, since the antigen and adjuvant are co-expressed as a fusion protein, optimization of their ratio is not feasible. The PCV2 Cap protein–flagellin complex vaccine developed in this study effectively resolves the issue of antigen-to-adjuvant ratio optimization. Even at a relatively low dosage of Nbcap-flagellin adjuvant (4 μg per mouse) and a reduced Cap protein vaccine dose (10 μg per mouse), with only two immunizations, sufficient protective effects were still observed following PCV2 challenge. We hypothesized that the production of adequate neutralizing antibodies is attributed to the fusion of flagellin with the Nbs, which may be associated with the high-affinity binding of flagellin to TLR5, enhancing cell activation, as well as the high antigen affinity and strong tissue penetration of the nanobody. Additionally, the generation of neutralizing antibodies may also involve other mechanisms, such as the assistance of T-cell help, B-cell activation, and regulation of adaptive immunity. Therefore, while the fusion of flagellin with Nbs may represent a potential strategy for enhancing immune responses, the specific mechanisms underlying this effect still require further experimental validation to clarify its potential application and efficacy in vaccine design.

The findings of this study indicate that the Nbcap-flagellin fusion protein holds significant potential for practical application and may serve as a cost-effective adjuvant for subunit vaccines against PCV2 infection, eliciting robust immunogenicity and therapeutic efficacy.

CRediT authorship contribution statement

Changjiang Wang: Writing – original draft, Validation, Funding acquisition. **Tianqi Xu:** Methodology, Data curation. **Jinliang Wang:** Validation. **Feng Li:** Validation, Funding acquisition. **Yu Guan:** Methodology. **Lin Dong:** Funding acquisition, Data curation. **Yan Wang:** Software, Investigation. **Weiqin Meng:** Validation. **Fengrong Tian:** Supervision, Resources. **Feng Wei:** Writing – review & editing, Methodology, Funding acquisition.

Informed consent statement

Not applicable.

Institutional review board statement

The animal study protocol was approved by the Ethics Committee of Shandong Binzhou Institute of Animal Husbandry and Veterinary Science (ASVMA-20111119-1).

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Declaration of competing interest

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.micpath.2025.107912.

Data availability

Data will be made available on request.

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