Enhancing immunogenicity and antiviral protection of inactivated porcine reproductive and respiratory syndrome virus vaccine in piglets

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OBJECTIVE
Porcine interferon-γ (poIFN-γ) and porcine granulocyte-macrophage colony-stimulating factor (poGM-CSF) are multifunctional cytokines that exhibit robust antiviral activity against porcine reproductive and respiratory syndrome virus (PRRSV). In this study, the immunoadjuvant effects of recombinant poIFN-γ-poGM-CSF fusion protein in inactivated PRRSV vaccine administered to piglets were assessed.

ANIMALS
Twenty-eight 4-week-old specific pathogen-free piglets.

METHODS
The experimental piglets were divided into control, highly pathologic PRRSV, PRRSV killed virus vaccine (KV), poIFN-γ-poGM-CSF, KV + 1.0 mg poIFN-γ-poGM-CSF, KV + 2.0 mg poIFN-γ-poGM-CSF, and KV + 4.0 mg poIFN-γ-poGM-CSF groups. A recombinant poIFN-γ-linker-poGM-CSF fusion gene was constructed via splicing by overlap extension PCR and prepared using an Escherichia coli expression system, after which its adjuvant activity in the context of PRRSV KV administration was assessed.

RESULTS
This analysis revealed the successful construction of the poIFN-γ-linker-poGM-CSF fusion gene via splicing by overlap extension PCR, with recombinant poIFN-γ-linker-poGM-CSF successfully being prepared in E. coli with a plasmid vector for expressing thioredoxin fusion proteins with an enterokinase site. Importantly, the coadministration of poIFN-γ-linker-poGM-CSF and PRRSV KV significantly increased neutralizing antibody titers, accelerated viral clearance, reduced clinical symptoms, and prevented highly pathogenic PRRSV infection.

CLINICAL RELEVANCE
The recombinant poIFN-γ-poGM-CSF fusion protein is a promising candidate adjuvant for use in the context of swine immunization and viral challenge.

Keywords: immunoadjuvant; piglets; poIFN-γ-poGM-CSF; PRRSV vaccine; recombinant fusion protein
periods in the lymphatic tissue following infection, and the production of neutralizing antibodies and cell-mediated immunity alone are insufficient to fully suppress PRRSV in the lungs or lymph nodes.12

Despite widespread vaccination programs implemented using both modified live virus and PRRSV killed vaccines (KV), PRRS has not been fully controlled owing to recombination and the high mutational frequency of the virus. Thus, effective, potent adjuvants should be identified to enhance the immunogenicity and protective efficacy of extant PRRSV vaccines. Traditional adjuvants include oil/water emulsions, liposomes, microparticles, and alum, which could be delivered to antigen-presenting cells (APCs), facilitating gradual, sustained immune stimulation by altering antigen conformational characteristics. Biological adjuvants can additionally drive APCs to secrete a range of cytokines, including Toll-like receptor agonists,12 chemical reagents,14–16 heat shock protein Gp96,17 bacterial products,18–20 and plasmid-based cytokine genes,19,21–25 which promote immune responses in the context of PRRSV vaccination.23 Interferon-γ (IFN-γ) is a pleiotropic type II interferon gene that plays central roles in coordinating both innate and adaptive immunity.26 Most IFN-γ is secreted by natural killer and activated T cells in response to pathogenic infection.27 The IFN-γ derived from natural killer cells and APCs can promote the upregulation of antiviral proteins within tissues in the early stages of a viral infection, followed by inhibiting viral replication and spread while simultaneously enhancing the phagocytic activity of phagocytes and promoting lymphocyte activation.28,29 Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a cytokine that promotes stimulatory protein expression by APCs. It further regulates Th1- and Th2-type CD4+ T-cell-mediated immune responses by shaping the activation of dendritic cells (DCs), which are a subset of APCs that are key coordinators of adaptive immunity.30 Furthermore, GM-CSF serves as a safe and effective immunostimulatory adjuvant in the context of viral vector-based vaccines, and studies have demonstrated that it could improve vaccinal immunogenicity.31 Previous studies have demonstrated that the in vitro and in vivo immunogenicities of PRRSV vaccines were enhanced by using recombinant porcine IFN-γ (poIFN-γ) and porcine GM-CSF (poGM-CSF). In this study, a recombinant fusion protein incorporating both of these cytokines was generated (poIFN-γ-linker-poGM-CSF) and expressed using the plasmid vector for expressing thioredoxin fusion proteins with an enterokinase site (pET32a vector) in an Escherichia coli–based expression system. The 2 cytokines were linked by the flexible peptide linker (Gly4Ser)3 to ensure that they did not affect each other’s spatial conformation. However, whether recombinant poIFN-γ-linker-poGM-CSF prepared using an E coli–based system exhibits adjuvant activity in the context of PRRSV vaccination has yet to be established. Therefore, this study focused on the preparation of poIFN-γ-linker-poGM-CSF, which was amplified via splicing by overlap extension PCR (SOE-PCR) before expression in E coli, with subsequent experiments to assess the adjuvant activity of the resultant fusion protein.

Methods

Plasmids, cells, and viruses

The prokaryotic pET32a(+) plasmid expression vector was purchased from a biological company (Takara Biotechnology Co Ltd). Wistar Institute Susan Hayflick (WISH) cells and Meat Animal Research Center-145 (MARC-145) cells were obtained from the same source (American Type Culture Collection). The highly pathologic PRRSV (HP-PRRSV) SX-TY strain (KY129875) was isolated and identified by our laboratory using MARC-145 cells and cultured for 5 generations. While the full HP-PRRSV-SX-TY genome sequence is not available at present, preliminary genome sequencing (GP5) suggests that this strain exhibits more than 99% nucleotide sequence homology with corresponding sequences of the standard HP-PRRSV JXA1 strain (EF112445).

Experimental animals and immunization grouping

Twenty-eight specific pathogen-free (PRRSV, classical swine fever virus [CSFV], porcine circovirus type 2 [PCV2], and African swine fever virus [ASFV]). 4-week-old piglets were purchased from a pig farm near Zhangjiakou, Hebei, after being screened to detect antibodies corresponding to these pathogens. The experimental piglets were fed independently during the study. Piglet serum samples were collected before the experiment to assess the PRRSV-specific antibodies, while PCR tests were applied to examine PRRSV, CSFV, PRRSV, PCV2, and ASFV viremia. The piglets were then randomly divided into 7 groups of 4, including control (group 1), highly pathogenic PRRSV (group 2), PRRSV KV (group 3), poIFN-γ-poGM-CSF (group 4), KV + 1.0 mg poIFN-γ-poGM-CSF (group 5), KV + 2.0 mg poIFN-γ-poGM-CSF (group 6), and KV + 4.0 mg poIFN-γ-poGM-CSF (group 7) groups. Piglets in groups 2 to 7 underwent IM immunization with inactivated PRRSV vaccine (1.0 X 106 TCID50/mL, 1 mL) and/or received a range of poIFN-γ-linker-poGM-CSFs (1, 2, and 4 mg/piglet). Piglets in the first (nonimmunized) group were instead administered an equivalent volume of PBS. At 21 days postimmunization (dpi), serum samples were collected from piglets in all groups and used for ELISA analyses of PRRSV-specific antibody induction. Piglets in all groups other than the first group were inoculated with the HP-PRRSV-SX-TY strain (1.0 X 106 TCID50/mL, 1 mL), with blood samples being collected at 0, 3, 5, 7, 14, and 21 days postchallenge (dpc). Rectal temperatures and mortality were recorded daily, and all surviving piglets were euthanized at 21 dpc, followed by necropsy and histopathological examination. The Animal Welfare Committee of Hebei Agricultural University approved all animal studies, with all pigs...
being monitored throughout the experimental period for clinical symptoms.

**Fusion gene construction of poIFN-γ-linker-poGM-CSF**

Polymerase chain reaction was used to amplify the poIFN-γ and poGM-CSF genes from blood samples using the following primers: poIFN-γ F1: ATGAGTTATACAACTTATTT; poIFN-γ F2: TTATTTTGATGCTCTGCCC; poGM-CSF F3: ATGTGGCTGCAGACCTGCT; and poGM-CSF F4: TTAATTCTTGACTGCC. These primer pairs specific for poIFN-γ and poGM-CSF were designed based on the known respective NM_213948.1 and DQ108393.1 sequences. After amplification, the poGM-CSF and poIFN-γ were separated via 1.5% agarose gel electrophoresis and purified using a PCR purification mini kit (China Biomed) according to the manufacturer’s instructions, after which they were sequenced. A flexible amino acid linker comprising 15 amino acids (12 glycine, 3 serine) was designed. Primer pairs for poIFN-γ-linker-poGM-CSF preparation were amplified from the genomic sequencing of poIFN-γ and poGM-CSF via gene SOE-PCR using the final 3 codons of poIFN-γ for codon optimization with the following primers: poIFN-γ-linker-poGM-CSF F1: CCGGAATTC (EcoRI) ATGAGTTATACAACTTATTT; F2: partial linker (reverse sequence) + TTTTGATGCTCTGCCC; F3: partial linker (forward sequence) ATGAGTTATACAACTGCT; and F4: CCGCTCGAG (XhoI) TTAATTCTTGACTGCC. These primers were synthesized by a biological company (Sangon Biotech). The poIFN-γ-linker and linker-poGM-CSF gene fragments were amplified from recovered agarose gel products (IFN-γ and GM-CSF) and served as PCR templates using the F1/F2 and F3/F4 primer pairs as per the instructions provided with the Pyrobest DNA Polymerase (Takara Biotechnology Co Ltd), respectively (Figure 1). The poIFN-γ-linker and linker-poGM-CSF PCR products were recovered using a low melting point agarose gel recovery kit (China Biomed).

**Recombinant fusion protein expression in E coli**

The E coli BL21 (DE3) competent cells were transformed with the pET32a-poIFN-γ-linker-poGM-CSF vector, and successfully transformed cells were identified via PCR and cultured overnight in 5 mL of Luria–Bertani (LB) medium supplemented with 100 μg/mL ampicillin at 37 °C. Next, 100 mL of fresh LB medium was added to a 500-mL flask containing the prepared cultures, followed by continued growth at 37 °C with constant shaking until samples reached an optical density at 600 to 800 nm of 0.6 to 0.8. The E coli BL21 (DE3) competent cells were transformed with the pET32a-poIFN-γ-linker-poGM-CSF vector, and successfully transformed cells were identified via PCR and cultured overnight in 5 mL of Luria–Bertani (LB) medium supplemented with 100 μg/mL ampicillin at 37 °C. Next, 100 mL of fresh LB medium was added to a 500-mL flask containing the prepared cultures, followed by continued growth at 37 °C with constant shaking until samples reached an optical density at 600 to 800 nm of 0.6 to 0.8.
at which time isopropyl β-D-1-thiogalactopyranoside (IPTG; final concentration: 1 mM) was added to induce polIFN-γ-linker-polGM-CSF expression. Following culture for an additional 6 hours, E. coli were harvested via centrifugation for 10 minutes at 10,000 X g at 4°C. Thereafter, 100 mg of the collected bacterial pellet was resuspended in 2 mL of suspension buffer (0.01 mM PBS, pH 7.2 to 7.4), and the resultant bacterial precipitate was lysed via ultrasonication (run for 10 seconds, stop for 10 seconds, and power at 70%) in an ice bath until the liquid was fully clarified. Samples were then centrifuged for an additional 10 minutes at 10,000 X g at 4°C, after which bacterial pellets were collected in PBS containing the recombinant polIFN-γ-linker-polGM-CSF protein, with SDS-PAGE being used to validate the presence of this fusion protein within these cells.

**Recombinant polIFN-γ-linker-polGM-CSF fusion protein purification**

Final precipitates were resuspended in binding buffer (20 mM Tris-HCl pH 7.9, 10 mM imidazole, 500 mM NaCl, and 8 M urea) and incubated overnight at 4°C, resulting in the release of periplasmic polIFN-γ-linker-polGM-CSF and other proteins into the surrounding media. Next, the samples were centrifuged for 20 minutes at 10,000 X g at 4°C, after which supernatants were collected and passed through a filter membrane (pore size, 0.22 μm). The resultant supernatant containing His-tagged polIFN-γ-linker-polGM-CSF was loaded onto a Ni-column at a flow rate of 10-fold the volume of Ni-agarose resin per hour. This column was washed with 15 volumes of Ni-agarose resin binding buffer, after which the bound polIFN-γ-linker-polGM-CSF protein was eluted using elution buffer (20 mM Tris-HCl pH 7.9, 500 mM imidazole, 500 mM NaCl, and 8 M urea) and dissolved in a urea concentration gradient (6, 5, 4, 2, 1, and 0 M) to facilitate protein renaturation, followed by dialysis against PBS (pH 8.0) to remove imidazole. A protein assay kit (Bradford Bio-Rad protein assay kit; Bio-Rad Laboratories) was then used to measure the protein concentrations in the prepared samples.

**Western Blotting**

Recombinant polIFN-γ-linker-polGM-CSF samples were separated via 12% SDS-PAGE and stained using either Coomassie Brilliant Blue G250 to assess protein purity or were transferred onto nitrocellulose membranes (Hybond-C; Amersham Pharmacia) for Western blotting. After transfer, membranes were blocked for 2 hours at room temperature (15 to 25°C) using 5% nonfat milk in TBS with Tween 20, followed by overnight incubation with mouse anti-His (1:1,500) at 4°C. Subsequently, blots were washed three times with PBS with Tween 20 (PBST; 5 min/wash) and incubated for 2 hours at room temperature with goat anti-mouse IgG-horseradish peroxidase (1:1,500). Blots were again washed with PBST, after which bands were detected using metallic-enhanced 3,3’-diaminobenzidine (Thermo Fisher Scientific), with signals being assessed using AlphaView 3.4 (ProteinSimple).

**Analysis of rpoIFN-γ-linker-polGM-CSF biological activity**

The biological activity of rpoIFN-γ-linker-polGM-CSF was measured using the human amniotic WISH cell line, which exhibits IFN-γ-dependent proliferation.

**Hematoxylin and eosin staining and lung scoring**

To determine whether the combination of PRRSV and the prepared adjuvant was associated with effective protection against HP-PRRSV challenge, we assessed lung tissue pathology in piglets immediately after death or following the euthanization of surviving piglets on day 21 postchallenge. Lung tissue samples were fixed in 10% neutral buffered formalin and then paraffinized and cut into sections for subsequent H&E staining to assess lung tissue pathology. Lesions in these samples were scored based on the observed severity of interstitial pneumonia (0, no lesion; 1, slight change; 2, scattered or partial interstitial damage; 3, 50% or less interstitial damage; 4, 51% to 75% damage with shrinking of the alveolar cavity; and 5, 76% to 100% damage consistent with severe interstitial pneumonia).

**Quantitative PCR**

A reagent (TRizol; Invitrogen) was used to extract total RNA from serum samples, after which PRRSV RNA was detected via quantitative PCR (qPCR) using the Real PCR PRRSV-2 RNA Mix (IDEXX). The resultant qPCR data were analyzed using a cycle threshold (Ct) cut-off value of 38 to reflect PRRSV RNA positivity, as per the manufacturer’s instructions.

**Enzyme-linked immunosorbent assay**

Serum samples were collected at the indicated time points from all piglets and used to measure anti-PRRSV antibody levels using the ELISA kit (IDEXX HerdChek PRRS X3 ELISA kit; IDEXX) according to the manufacturer’s instructions.

**Virus neutralization assay**

To confirm the ability of PRRSV-neutralizing antibodies present within serum samples to bind to the virus and block subsequent infection, MARC-145 cells were utilized to conduct a virus-neutralization assay. Briefly, serum samples were thermally inactivated for 30 minutes at 56°C, followed by 2-fold serial dilution in cell maintenance solution. An equal volume of HP-PRRSV-SX-TY was then added to each well, followed by incubation for 1 hour at 37°C to allow antibodies sufficient time to bind to the virus. The solutions were then transferred onto MARC-145 cell monolayers cultured in 96-well plates, and cells were incubated for 72 hours under normal culture conditions. Cytopathic effects were then identified by examining the cultured cells, and neutralizing antibody titers in these serum samples were calculated based on end-point titers.

**Statistical analysis**

GraphPad Prism v 9.0 (GraphPad Software) was used to conduct all statistical analyses. Continuous variables are described using mean ± SD, and
intergroup comparisons were conducted using analysis of 1-way ANOVA. Bonferroni correction was applied for pairwise comparisons in case of statistically significant differences. For categorical data, description is based on median and interquartile range, and intergroup comparisons were performed using nonparametric tests. For all outcomes, statistical significance was defined as having 2-sided $P$ values less than .05.

Results

Successful poIFN-$\gamma$-linker-poGM-CSF prokaryotic expression vector construction

The IFN-$\gamma$ and GM-CSF genes were successfully amplified from porcine blood samples using PCR, and then the poIFN-$\gamma$-linker-poGM-CSF construct was amplified using recovered poIFN-$\gamma$ and poGM-CSF gene products via the prepared poIFN-$\gamma$-linker and linker-poGM-CSF primer pairs using SOE-PCR (Figure 2). The gene products were then recovered following agarose gel electrophoresis and subcloned into the pET32a(+) vector to yield a pET32a-poIFN-$\gamma$-linker-poGM-CSF. The integrity of this plasmid was then validated through targeted genetic sequencing, and its structure was confirmed via restriction enzyme digestion. The analysis revealed that the poIFN-$\gamma$-linker-poGM-CSF gene was successfully fused with a 6X His-tag at its 3’ terminus.

Preparation and validation of recombinant poIFN-$\gamma$-linker-poGM-CSF

To confirm the secretion and large-scale preparation of recombinant poIFN-$\gamma$-linker-poGM-CSF, BL21 E coli were transformed with the synthesized pET32a-poIFN-$\gamma$-linker-poGM-CSF plasmid. Positive transformants were then screened on LB agar plates containing 100 μg/mL ampicillin, with expression levels compared using SDS-PAGE (Figure 3) and Western blotting. The recombinant poIFN-$\gamma$-linker-poGM-CSF protein was detectable in both supernatants and inclusion body precipitants following IPTG (1 mM) induction at 37°C, with the highest levels of expression primarily being detectable in inclusion body precipitates. poIFN-$\gamma$-linker-poGM-CSF protein expression levels differed across the tested time points (2, 4, 6, and 8 hours), with maximum expression being observed 6 hours after IPTG induction. These data confirmed that this E coli-based system was sufficient to facilitate the secretion of recombinant poIFN-$\gamma$-linker-poGM-CSF in a manner amenable to large-scale production, with yields as high as 50 mg/L in fermentation media. After incubation with the IFN-$\gamma$-dependent WISH human amniotic cell line to assess the biological activity of the poIFN-$\gamma$ protein within the prepared recombinant poIFN-$\gamma$-linker-poGM-CSF fusion protein, anMTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay was performed, revealing that, much like recombinant human IFN-$\gamma$ (Thermo Fisher Scientific) and eukaryotic cell–derived poIFN-$\gamma$-linker-poGM-CSF, the recombinant poIFN-$\gamma$-linker-poGM-CSF prepared at scale in E coli could also effectively stimulate WISH cell proliferation.

Immunization schedules

Piglets were immunized with PRRSV KV and/or recombinant poIFN-$\gamma$-linker-poGM-CSF before viral inoculation (Figure 4). Briefly, piglets were inoculated with PRRSV KV vaccine and appropriate recombinant poIFN-$\gamma$-linker-poGM-CSF doses via the same route at −28 dpc. The blood samples of all piglets were collected at −7 dpc. On day 0, piglets...
were challenged via the same route with HP-PRRSV-SX-TY (1 mL of viral stock; 1.0 X 10^6 TCID_{50}/mL). Thereafter, blood samples were collected from all immunized piglets and PBST-treated controls at the indicated time points 21 dpc, and humoral immunity was analyzed using ELISA. The sample-to-positive ratio (S:P ratio) was calculated to determine the presence of anti-PRRSV antibodies. The S:P ratio is defined as: S:P = (sample A(650)-NCx)/(PCx-NCx). Anti-PRRSV antibodies were detectable in all animals other than PBS controls at this time point, consistent with seroconversion, indicating that poIFN-γ-linker-poGM-CSF coadministration with PRRSV KV induced robust antibody-based immune responses to PRRSV.

**Recombinant poIFN-γ-linker-poGM-CSF functions as an adjuvant and protects piglets from HP-PRRSV challenge**

Piglets in the unvaccinated group began to die within 9 dpc, and all animals in this group had died as of 19 dpc (Figure 5). In contrast, a 100% survival rate was observed in piglets in the

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**Figure 3**—A—Isopropyl β-D-1-thiogalactopyranoside (IPTG) was used to induce the expression of the porcine interferon-γ (poIFN-γ)-linker-porcine granulocyte-macrophage colony-stimulating factor (poGM-CSF) protein for different periods, after which it was detected by 10% SDS-PAGE and Coomassie Blue staining. Lane 1 = pET32a empty vector. Lane 2 = pET32a/poIFN-γ-linker-poGM-CSF vector samples at 0 h following IPTG induction. Lane 3 = pET32a/poIFN-γ-linker-poGM-CSF vector samples at 2 h following IPTG induction. Lane 4 = pET32a/poIFN-γ-linker-poGM-CSF vector samples at 4 h following IPTG induction. Lane 5 = pET32a/poIFN-γ-linker-poGM-CSF vector samples at 6 h following IPTG induction. Lane 6 = pET32a/poIFN-γ-linker-poGM-CSF vector samples at 8 h following IPTG induction. M = Protein markers. B—Assessment of poIFN-γ-linker-poGM-CSF protein production by different pET32a/poIFN-γ-linker-poGM-CSF vector-transformed bacterial samples following IPTG induction at different time points as assessed via 10% SDS-PAGE and Coomassie Blue staining. Lane 1 = pET32a empty vector. Lane 2 = poIFN-γ-linker-poGM-CSF in supernatant samples. Lane 3 = poIFN-γ-linker-poGM-CSF in inclusion bodies. M = Protein markers. C—poIFN-γ-linker-poGM-CSF protein detection via Western blotting. Lane 1 = Purified poIFN-γ-linker-poGM-CSF protein pET32a/poIFN-γ-linker-poGM-CSF vector-transformed bacteria treated with Ni-NTA agarose beads. M = Protein markers. D—analysis of poIFN-γ-linker-poGM-CSF biological activity based on the ability of the recombinant protein to promote Wistar Institute Susan Hayflick (WISH) cell proliferation as detected in an MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; Sigma) assay; horizontal bars above the plots indicate results that differed significantly between groups. BSA = Bovine serum albumin.
Figure 4—A—At −28 d postchallenge, piglets in all groups other than the PBS control and mock groups were IM immunized with a porcine reproductive and respiratory syndrome virus (PRRSV) killed virus vaccine (KV) and/or indicated amounts of recombinant porcine interferon-γ (poIFN-γ)-linker-porcine granulocyte-macrophage colony-stimulating factor (poGM-CSF). At −7 d postchallenge, blood samples were collected from all piglets. At 0 d postchall enge, piglets in the immunization groups were challenged with highly pathologic PRRSV (HP-PRRSV)-SX-TY via the same administration route (1 mL of viral stock; 1.0 \times 10^6 TCID/mL). Following challenge, blood samples were collected from piglets at the indicated time points through 21 d postchallenge. B—Serum samples were collected from piglets in all groups other than the PBS control group at −7 d postchallenge to assess seroconversion using an ELISA kit (IDEXX HerdChek PRRS X3 ELISA kit). Experimental analyses were repeated a minimum of 3 times per serum sample, and data are presented as means ± SD; horizontal bars above the bar graph indicate results that differed significantly between groups. NS = Not significant.* P < .05.

polFN-γ-linker-poGM-CSF– or PRRSV KV–immunized groups following HP-PRRSV-SX-TY challenge. The rectal temperatures of these animals were also monitored daily to assess their clinical symptoms. Peak temperatures were observed at 4 dpc in all animals other than PBS controls, with recovery to normal temperatures by 9 dpc in the combined medium- and high-dose recombinant polFN-γ-linker-poGM-CSF + PRRSV KV–immunized groups. In contrast, a slightly higher peak temperature was observed at 4 dpc in the
low-dose recombinant poIFN-γ-linker-poGM-CSF + PRRSV KV–immunized group, with animals ultimately recovering by 11 dpc. During the peak fever period, the rectal temperatures of piglets in the recombinant poIFN-γ-linker-poGM-CSF–treated groups or PRRSV KV–immunized groups were significantly higher than those of piglets in the recombinant poIFN-γ-linker-poGM-CSF + PRRSV KV–immunized groups. This suggests that the use of recombinant poIFN-γ-linker-poGM-CSF as an adjuvant in the context of PRRSV KV immunization was associated with improvements in PRRSV symptoms compared with using either one of these agents in isolation.

**Recombinant poIFN-γ-linker-poGM-CSF significantly reduces pathological lung lesion severity following HP-PRRSV challenge**

Piglets were either necropsied immediately following death or euthanized at the end of the experiment to assess pulmonary pathology associated with HP-PRRSV challenge (Figure 6). The most prominent causes of death in piglets included pulmonary inflammation, diffuse interstitial pneumonitis, and pulmonary edema with extensive bleeding. Lung histopathological scoring was applied to quantify the pathological changes in PRRSV-infected animals. This analysis revealed that piglets challenged with HP-PRRSV-SX-TY exhibited significantly higher scores than animals in any other groups. We noted adjuvant poIFN-γ-linker-poGM-CSF enhanced the immunological efficacy of PRRSV KV immunization, protecting piglets against severe lung damage following HP-PRRSV challenge. In contrast, the histopathological scores for piglets in the medium- or high-dose recombinant poIFN-γ-linker-poGM-CSF + PRRSV KV–immunized groups did not significantly differ from those of control piglets.

**Recombinant poIFN-γ-linker-poGM-CSF protects immunized piglets against viremia following HP-PRRSV challenge**

Quantitative PCR was used to analyze PRRSV RNA levels in serum samples from piglets in each
Beginning at 3 dpc, Ct values for PRRSV RNA in these samples reached their lowest levels (22 to 24) as a consequence of HP-PRRSV-SX-TY challenge. Seven days postchallenge, these Ct values trended upward over time in the vaccinated groups. Moreover, relative to unvaccinated piglets, all vaccinated piglets (recombinant poIFN-γ-linker-poGM-CSF and/or PRRSV KV) exhibited varying levels of viremia amelioration. Importantly, piglets in the medium- and high-dose recombinant poIFN-γ-linker-poGM-CSF + PRRSV KV-immunized group maintained Ct values greater than 38 (consistent with a negative result) from 14 dpc until the end of the study, consistent with the elimination of PRRSV viremia in these animals. Taken together, these data indicate that recombinant poIFN-γ-linker-poGM-CSF administration in combination with PRRSV KV immunization was sufficient to decrease PRRSV viremia in piglets following HP-PRRSV challenge.

**Recombinant poIFN-γ-linker-poGM-CSF promotes enhanced neutralizing antibody production**

A neutralization assay was conducted using the same HP-PRRSV-SX-TY virus to explore the ability of recombinant poIFN-γ-linker-poGM-CSF treatment to elicit higher titers of PRRSV-specific neutralizing antibody (NAb) production (Supplementary Figure S2). Serum NAb levels were negligible...
irrespective of the PRRSV strain used for immunization before 0 dpc, since PRRSV induces poor NAb responses. Beginning at 5 dpc, however, NAb titers in serum samples rose, with the highest levels being observed in the medium- and high-dose recombinant poIFN-γ-linker-poGM-CSF + PRRSV KV-immunized groups, wherein these levels remained elevated to the end of the experimental period at 21 dpc. In contrast, viremia shedding levels (data expressed as qPCR Ct values) of PRRSV KV-vaccinated or recombinant poIFN-γ-linker-poGM-CSF-vaccinated piglets were markedly lower than those for both the PRRSV KV- and recombinant poIFN-γ-linker-poGM-CSF-vaccinated groups. In contrast, those for the PRRSV KV- and recombinant poIFN-γ-linker-poGM-CSF-vaccinated (medium- or high-dose group) piglets were markedly higher than those for both the PRRSV KV- and recombinant poIFN-γ-linker-poGM-CSF-vaccinated (low-dose group) groups. Collectively, these results confirmed that recombinant poIFN-γ-linker-poGM-CSF administration as an adjuvant in the context of PRRSV KV immunization was sufficient to evoke enhanced NAb responses to HP-PRRSV challenge.

Discussion

Genetically engineered human IFN-γ and GM-CSF are widely used in clinical practice but not in veterinary practice. This study linked poIFN-γ and poGM-CSF proteins together with a flexible linker domain using an SOE-PCR approach, yielding an intact fusion protein. After producing this recombinant poIFN-γ-linker-poGM-CSF protein in an E coli expression system, it was used as an adjuvant to enhance the immunization outcomes of PRRSV KV in piglets. The current investigation demonstrated that the administration of recombinant poIFN-γ-linker-poGM-CSF exhibited potent immunological activity, augmenting humoral immune response against PRRSV KV. This resulted in effective protection against viremia subsequent to HP-PRRSV challenge and prevented the HP-PRRSV infection.

Virus-carrying piglets and finishing pigs are important sources of persistent PRRSV infection in swine herds, and inactivated vaccines have proven ineffective as a means of controlling such diseases.2 Previous studies33 indicated that inactivated vaccine administration combined with immune adjuvants could enhance antibody production, lymphocyte proliferation, and cellular immunity following immunization. Moreover, piglets treated with inactivated vaccines combined with immune adjuvants were associated with improved clinical symptoms following PRRSV challenge.35 These results could be attributed to adjuvants promoting APC activation while also enhancing the secretion of certain cytokines and maintaining natural antigen conformations to enhance their presentation by APCs, facilitating gradual, sustained immune stimulation. Our study developed a fusion protein adjuvant aimed at overcoming the shortcomings of PRRSV KV vaccination and evaluated the immunoadjuvant effects of recombinant poIFN-γ-poGM-CSF fusion protein on the inactivated PRRSV vaccine in piglets.

Interferon-γ secreting cells, which primarily consist of CD4+CD8+ cells and some CD4-/CD8αβ- cytotoxic T cells, are capable of promoting antiviral immunity by upregulating major histocompatibility complex molecules and other key proteins with direct antiviral activity, including IFN-stimulated genes.34–36 Interferon-γ-mediated antiviral immunity is related to the target cell types, and response to type I and II IFN signaling is affected by distinct signaling cascades. Viral entry through interference with endosomal membrane viral fusion could be disrupted by proteins in the IFN-induced transmembrane (IFITM) family of IFN-stimulated genes, especially IFITM3.37–40 Interferon-γ levels were elevated following vertical PRRSV infection in piglets, and immunoregulatory activity could be affected by sustained IFN-γ expression and reduced IL-10 production.41 Moreover, an increase in the number of IFN-γ-positive cells and a decrease in macrophage count were observed in PRRSV-infected animals, which might be explained by lung injury and persistent lung PRRSV infection.42 Thus, IFN secretion may be insufficient to activate innate immunity in host species, and other proinflammatory cytokines should be combined to elicit effective antiviral immunity in PRRSV-infected animals. The GM-CSF is a potent cytokine that presents immunomodulatory activity and a good safety profile. It could also induce monocyte differentiation and promote the development of bone marrow-derived dendritic cells, further promoting costimulatory molecule expression by T cells. Studies43,44 have demonstrated that GM-CSF could be considered an immune adjuvant, which could be used in various vaccinal contexts with low toxicity levels. Dendritic cells, as APCs, play an important role in inducing innate and adaptive immune responses. The GM-CSF can regulate Th1 and Th2 immune responses through its ability to promote DC activation and maturation.45 Moreover, the costimulatory molecule expression of APCs was increased by GM-CSF.46

The pET32a prokaryotic expression vector enables the expression of fusion proteins containing 6 histidine ligand tags. In E coli, recombinant proteins primarily exist in both soluble and insoluble forms, with the latter commonly being present within inclusion bodies.47 The process underlying inclusion body formation is complex and depends on the rates of cytoplasmic protein production as noncrystalline aggregates; amorphous proteins can form when the time to permit protein folding is insufficient. Moreover, this process could be affected by other factors, including pH, temperature, media composition, and ionic strength.48 The present study successfully amplified poIFN-γ-linker-poGM-CSF via SOE-PCR using full-length poIFN-γ and poGM-CSF genes as templates, subsequently achieving the successful secretory expression of this recombinant fusion protein in E coli BL21(DE3) using the pET32a(+) expression vector. We then purified this recombinant fusion protein through steps, including
urinary, low-concentration renaturation, solution-based renaturation, and PBS dialysis, followed by the use of nickel affinity chromatography. The SDS-PAGE and Western blotting assays were subsequently used to detect the resultant fusion protein, which was found to be nontoxic and to exhibit good biological activity following purification.

As multifunctional cytokines, IFN-γ and GM-CSF could be applied as potential vaccine adjuvants to protect against infectious disease. However, whether the combination of poIFN-γ-linker-poGM-CSF and an inactivated vaccine can synergistically protect against disease through enhanced immunomodulation remains unclear. The current study constructed a poIFN-γ-linker-poGM-CSF fusion gene via a SOE-PCR approach using amplified poIFN-γ and poGM-CSF, after which an E. coli expression system was used to successfully purify the resultant recombinant poIFN-γ-linker-poGM-CSF protein. Considering that the nonprotective PRRSV-specific NAb responses in piglets could be induced by PRRSV KV vaccination, the kinetic analyses of PRRSV-specific NAb production are responsible for the latest emergence of highly pathogenic porcine reproductive and respiratory syndrome virus in China. Our study found that using poIFN-γ-linker-poGM-CSF as an adjuvant in the context of PRRSV KV immunization could enhance the activation of humoral immune responses, driving robust antibody production and effectively improving porcine survival in response to PRRSV challenge; reducing clinical symptoms, fever, and tissue pathology; and expediting viral clearance in animals that underwent combination treatment.

This study presented a recombinant poIFN-γ-linker-poGM-CSF, generated using an E. coli-based expression system, which could enhance the immunogenicity and protective efficacy of PRRSV KV immunization by inducing high NAb titers. Therefore, the recombinant poIFN-γ-linker-poGM-CSF is considered a promising candidate adjuvant for swine immunization and viral challenge.

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Disclosures

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**Supplementary Materials**

Supplementary materials are posted online at the journal website: avmajournals.avma.org.