Supplementary methods

1. cDNA amplification

Viral RNA was extracted from the Chinese isolate HH08 of PRRSV using TRIzol reagent (Invitrogen, USA) and viral cDNA was synthesized using Oligo dT primer (TaKaRa, China) according to the manufacturer's instructions. According to the GP5 sequence of PRRSV (GenBank Accession number: GQ184821), primers were designed for amplifying a fragment covering the ORF5 gene of PRRSV (Supplementary Table 1). PCR was carried out using PrimeSTAR HS DNA Polymerase (TaKaRa, China) in a 50 μl reaction volume consisting of 1× PrimeSTAR Buffer (Mg^{2+} plus), 200 μM of each dNTP, 0.2 μM of forward and reverse primers, 1.25 U of PrimeSTAR HS DNA Polymerase and ~200 ng of viral cDNA template. PCR amplifications were performed as follows: 1 cycle of 98°C for 5 min, then 25 cycles of 98°C for 10 s, 60°C for 15 s, and 72°C for 90 s, followed by 1 cycle of 72°C for 10 min. The amplified PCR product was purified, cloned into pCR2.1-T with a TA Cloning Kit (Invitrogen, USA), and sequenced by Genscript (Nanjing, China).

2. Construction of recombinant plasmid

The GP5 ectodomain with the deletion of its signal peptide and transmembrane regions was designated according to a reference with minor modifications [Ren et al., 2010]. The truncated GP5 was amplified by overlap-PCR with two pairs of primers (Supplementary Table 1). *Eco*RI and *Xho*I restriction enzyme sites were introduced in GP5a-F and GP5b-R, respectively (underlined parts). A linker sequence encoding two repeated amino acid sequences (GGGGS) was introduced into primers GP5a-R and GP5b-F (The linker sequence was in black bold). GP5a and

GP5b fragments were amplified by PCR using GP5a-F/GP5a-R and GP5b-F/GP5b-R respectively as described above with extension for 30 s at 72°C. The resulting PCR products were electrophoresed on 1% agarose gels and purified as the template of the truncated GP5 gene amplification using primers GP5a-F and GP5b-R. The amplified products were extracted from single bands, purified, and cloned into the pGEM-T Cloning Vector (Promega, USA) designated as pGEM-GP5ab for sequencing by Genscript.

3. Expression and purification of PRRSV GP5

The GP5ab fragment was inserted into the *Eco*RI and *Xho*I digested expression vector pET-32a or pGEX-6p-1 to create pET32a-GP5ab or pGEX-6p-1-GP5ab respectly. Recombinant plasmid pET32a-GP5ab or pGEX-6p-1-GP5ab was purified with a plasmid purification kit (TaKaRa, China) and transformed into host cells, *Escherichia coli (E. coli)*, BL21(DE3) pLysS. Positive clones were identified on Luria-Bertani (LB) agar plates containing ampicillin (100 μg/ml). Protein expression was optimized according to a recent reference with minor modification [Liu et al., 2009]. Briefly, the *E. coli* harboring pET32a-GP5ab or pGEX-6p-1-GP5ab was cultured in LB liquid medium at 37°C with shaking until the optical density (OD) of the culture at 600 nm reached 0.6. Then, isopropyl-β-dthiogalactoside (IPTG) was added to a final concentration of 0.5 mM to induce expression at 25°C for 5 h. The empty vector transformed bacteria were used as control. The bacteria were pelleted at 10,000 rpm, at 4°C for 10 min and re-suspended in TE buffer (50 mM Tris and 1 mM EDTA, pH8.0). Then, they were digested with lysozyme at a final concentration of 100 μg/L at room temperature for 30 min. The cell suspension was sonicated on ice for 30 min. Then the lysate was centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant and the pellets were mixed with sodium dodecyl sulfate

(SDS)-loading buffer, respectively. Both samples were subjected to 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The purification of His-GP5 inclusion bodies were performed by using His•Bind Purification Kit (Novagen, USA) under the condition of 6 M urea. The purified protein of interest was designated as His-GP5.

4. Purification of Salmonella typhimurium FliC flagellin

Native FliC was purified from the attenuated *S.* Typhimurium SL7207 strain expressing FliC according to a reference with minor modification [Ibrahim et al., 1985]. Briefly, bacteria were grown in LB overnight at 37°C under aeration (80 rpm). Cells were washed once with phosphate-buffered saline (PBS) and then adjusted to pH 2.0 with 1 M HCl and maintained at that pH under constant stirring for 30 min at room temperature. The cell suspensions were centrifuged to remove the cells and the pH of the supernatant was adjusted to 7.2 with 1 M NaOH. The flagellar filaments were collected from the supernatant following ammonium sulfate precipitation. The precipitate, which contained polymerized flagellin, was dissolved in approximately 5 ml of distilled water and then transferred to dialysis tubing which had a molecular weight cutoff of 50,000 (Spectrum Medical Industries, USA). The protein content of FliC was determined with the Bradford assay and by SDS-PAGE analyses.

5. Western blotting

The product expressed from BL21(DE3)(pET32a-GP5) and extracted FliC were subjected to SDS-PAGE and then transferred to a nitrocellulose membrane, respectively. The membranes were blocked with blocking buffer (5% non-fat dry milk and 0.05% Tween-20 in PBS) at 4°C overnight. The next day, the membranes were incubated with a polyclonal antibody against PRRSV or FliC

(1:1000 diluted in PBS-0.05% Tween 20, PBST) at 37°C for 2 h. After washing three times with PBST, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:5000 diluted in PBST, Boster, China) at 37°C for 1 h. The protein bands were visualized via diaminobenzidine enzyme-based color development in the dark and terminated by distilled water.

6. Endotoxin removal of GP5 and FliC

Contaminating lipopolysaccharide (LPS) was removed from the recombinant protein His-GP5 and FliC by using the ProteoSpin™ Endotoxin Removal Kit Maxi for protein & peptides (Norgen, Canada), and the residual LPS content of the protein was measured using a chromogenic end-point tachypleus amebocyte lysate (CE TAL) assay kit (Chinese Horseshoe Crab Reagent Manufactory Co., Ltd., Xiamen, China) according to the manufacturer's instructions.

7. Administration of flagellin to conscious mice

6-week-old female C57BL/6 mice were purchased from Comparative Medicine Centre of Yangzhou University. They were housed in isolators and fed with a pathogen-free diet and water. The procedures described in this study were approved by the Committee on the Ethics of Animal Experiments of Yangzhou University, Yangzhou, China. C57BL/6 mice were injected intraperitoneally with prepared endotoxin-free FliC, given at a dose of 2 μg/mouse. At selected time-points (0 h, 1 h, 2 h, 6 h and 12 h) post injection, 5 mice were sacrificed by pentobarbital overdose each time, and the spleen was removed for subsequent mRNA detection of TLR5 and inflammatory cytokines.

8. RNA isolation, RT-PCR and quantitative real-time PCR

Tissues were homogenized in TRIzol reagent (Invitrogen), and total RNA was prepared as directed by the manufacturer. RNA concentrations were determined by spectrophotometer readings at 260 nm. Quantitative real-time PCR (qRT-PCR) was performed to measure mRNA expression levels of TLR5, IL-6, TNF-α, IL-1β, IL-12 and IL-10 using SYBR Premix Ex Taq II (Perfect Real Time; TaKaRa Biotechnology, Dalian, China) using an ABI 7500 real-time detection system (Applied Biosystems, Carlsbad, CA) with designed primers (Supplementary Table 1). Amplification was performed in a total volume of 20 μl containing 10 μl of 2×SYBR Premix Ex Taq II, 2 μl of the diluted cDNA, and 0.8 μl of each primer. The real-time PCR program started with denaturing at 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 34 s. Dissociation analysis of amplification products was performed at the end of each PCR to confirm that only one PCR product was amplified and detected. Data were analyzed with ABI 7500 SDS software (ABI), with the baseline being set automatically by the software. The threshold method was used for quantification of the mRNA level [Livak and Schmittgen, 2001] and ΔC_T values were calculated on the basis of the internal standard GAPDH signal. Results were expressed as $2^{-\Delta\Delta CT}$ (n-fold change compared to the 0 h group).

9. Mice immunization

To test whether the mixture vaccination of GP5 and FliC provides greater immune response, C57BL/6 mice were randomly divided into four groups (5 mice per group) and immunized intraperitoneally either with GP5, GP5 + FliC, GP5 + aluminium adjuvant (Thermo, USA), or PBS, respectively. These mice were immunized three times on days 1, 15, and 29 at a dose of 50 μg GP5, 2 μg FliC or isochoric aluminium adjuvant in 200 μL. Blood was collected from eye sockets after 4 and 6 weeks of primary immunization and sera were stored at −70°C until they

were tested by ELISA for antibodies and cytokines.

10. Determination of anti-GP5 antibodies

Serum IgG titers to GP5 were measured by ELISA according to a reference with minor modification [Cui et al., 2012]. Briefly, 96-well microtiter plates were coated with the recombinant GST-GP5 (0.5 ug/ml) in 50 mM carbonate buffer (pH 9.6) at 4°C overnight and blocked for 2 h at 37°C with 1% BSA in PBST. After washing three times with PBST, sera were added in a dilution of 1:100 with a two-fold dilution series in the blocking buffer and incubated for 2 h at 37°C. After five washes with PBST, Antigen specific antibodies were detected using goat anti-mouse IgG conjugated to horse-radish peroxidase (1:10000 dilution) for 1 h at 37°C. The ELISA was developed using TMB and H_2O_2 as substrates, and optical density was read at 450 nm (A450) with an ELISA reader (Bio-TekEL 680, USA).

11. Detection of sera IL-4 and IFN-γ cytokines

The levels of the cytokines (IL-4 and IFN-γ) in serum of each mouse were detected by commercially available ELISA kits (BD Pharmingen, USA) according to the manufacturer's protocol. Six standards (with expected concentrations of 1,000, 500, 250, 125, 62.5, 31.25 and 15.65 pg/ml) were prepared by serial dilution with an assay buffer kit, using a sensitivity (threshold for detection) of 8.0 pg/ml. The prepared plates were analyzed using a microplate reader at 450 nm.

12. Statistical analysis

The significance of the difference between groups injected with FliC at different time points, or immunized with mixed proteins and GP5 alone was determined by Student's t-test with Instat

version 5.0 (GraphPad Software, San Diego, CA). Statistical significance was determined at $P < 0.05$ (*), $P < 0.01$ (**) or $P < 0.001$ (***).

References

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Ibrahim GF, Fleet GH, Lyons MJ, Walker RA: Method for the isolation of highly purified *Salmonella* flagellins. J Clin Microbiol 1985; 22: 1040-1044.

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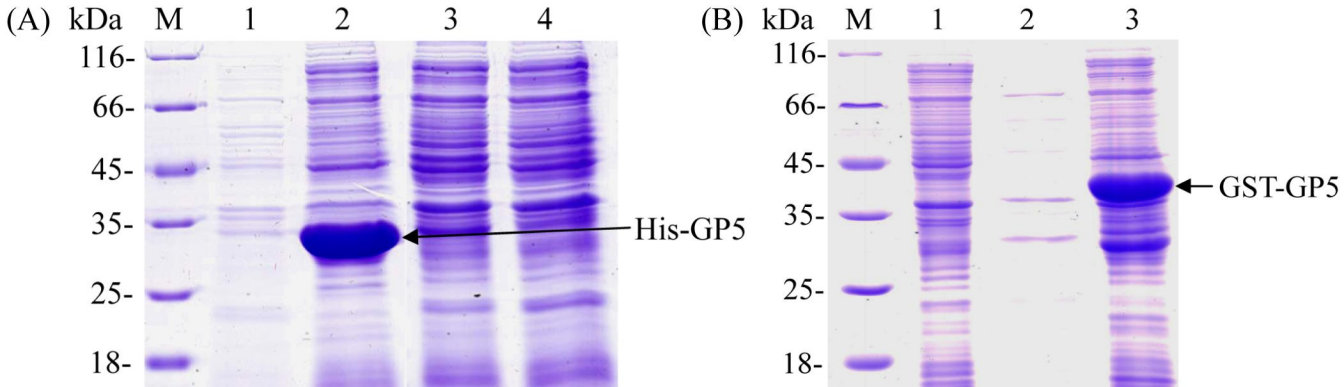
Supplementary figure legends

Supplementary Fig. 1. SDS-PAGE analysis of product expressed by recombinant bacteria of BL21 (DE3)(pET32a-GP5) (left) and BL21(DE3)(pGEX-6p-1-GP5) (right). The bacteria harboring pET32a-GP5 or pGEX-6p-1-GP5 were induced with 0.5 mM IPTG at 25°C for 5 h, and the bacterial protein were analyzed by SDS-PAGE respectively. (A) Lanes: M, molecular weight markers; 1, Lysate supemaniant of BL21(DE3)(pET32a-GP5) induced by IPTG; 2, Inclusion bodies of BL21(DE3)(pET32a-GP5) induced by IPTG; 3, product of BL21(DE3)(pET32a-GP5) not induced; 4, product of BL21(DE3)(pET32a) induced by IPTG. (B) Lanes: M, molecular weight markers; 1, product of BL21(DE3)(pGEX-6p-1) induced by IPTG; 2, Lysate supemaniant of BL21(DE3)(pGEX-6p-1-GP5) induced by IPTG; 3, Inclusion bodies of BL21(DE3)(pGEX-6p-1-GP5) induced by IPTG.

Supplementary Fig. 2. Levels of TLR5 (A), IL-6 (B), TNF-α (C), IL-1β (D), IL-12 (E) and IL-10 (F) mRNAs in spleen of mice following injection with prepared endotoxin-free FliC. Female C57BL/6 mice were immunized intraperitoneally with prepared endotoxin-free FliC, given at a dose of 2 μg/mouse. Data shown are the fold changes in mRNA expression compared with that in 0 h controls, based on five mice for each time and determined by qRT-PCR. Error bars indicate standard deviations of the means. Statistical significance was determined at $P < 0.05$ (*) or $P < 0.001$ (***).

Supplementary Fig. 3. Detection of sera IL-4 and IFN-γ cytokines after the third immunization. Female C57BL/6 mice were immunized intraperitoneally either with recombinant protein alone (50 μg His-GP5), admixed with flagellin (50 μg His-GP5 + 2 μg FliC), or admixed with aluminium (50 μg His-GP5 + isochoric Alum) on days 1, 15, 29, and bled on day 42 for analysis of IL-4 and IFN-γ cytokines by sandwich ELISA. Columns represent the mean ± SD of three independent experiments. Asterisks indicate significant differences compared to C57BL/6 mice immunized with GP5 alone by using the Student t test ($P < 0.01$).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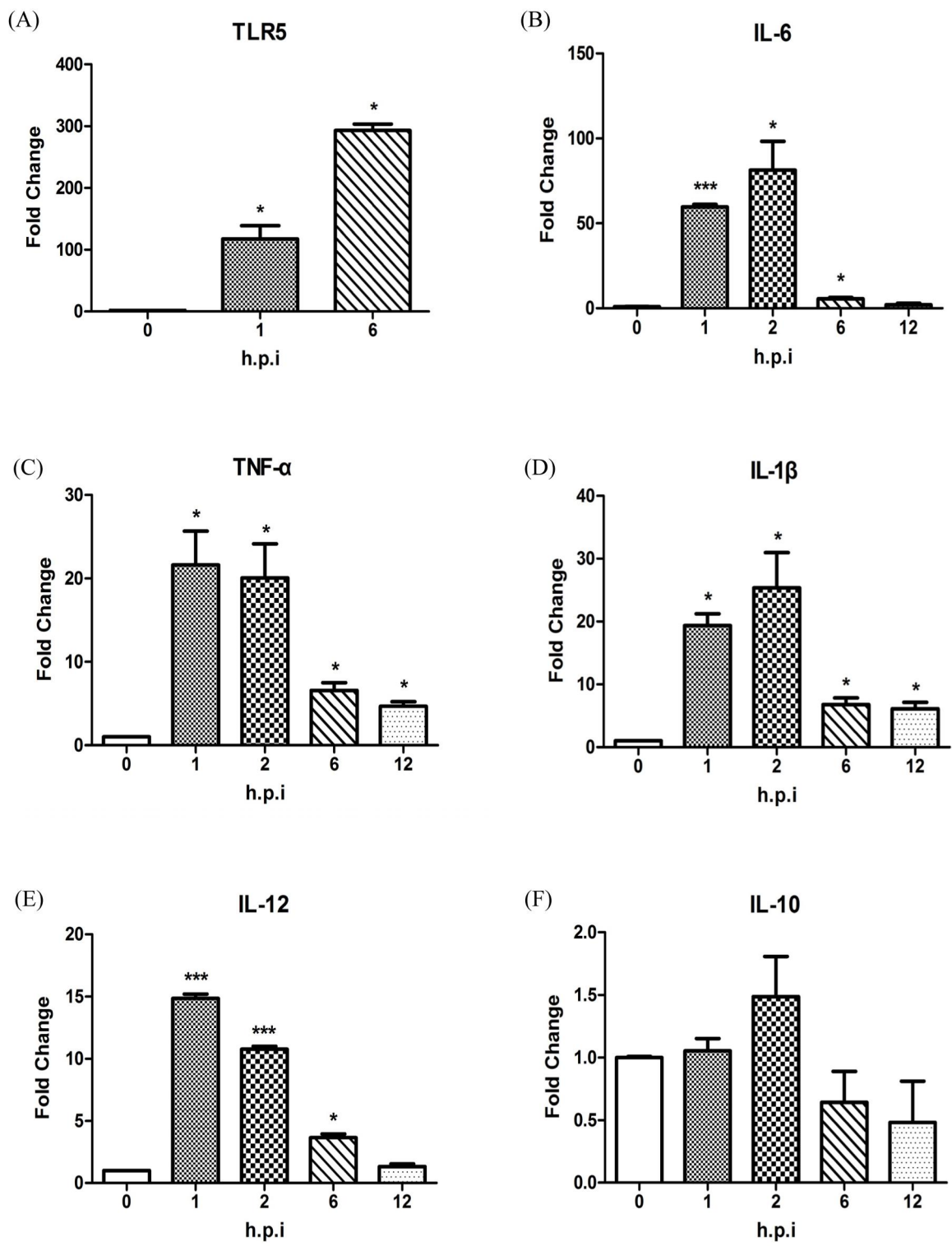
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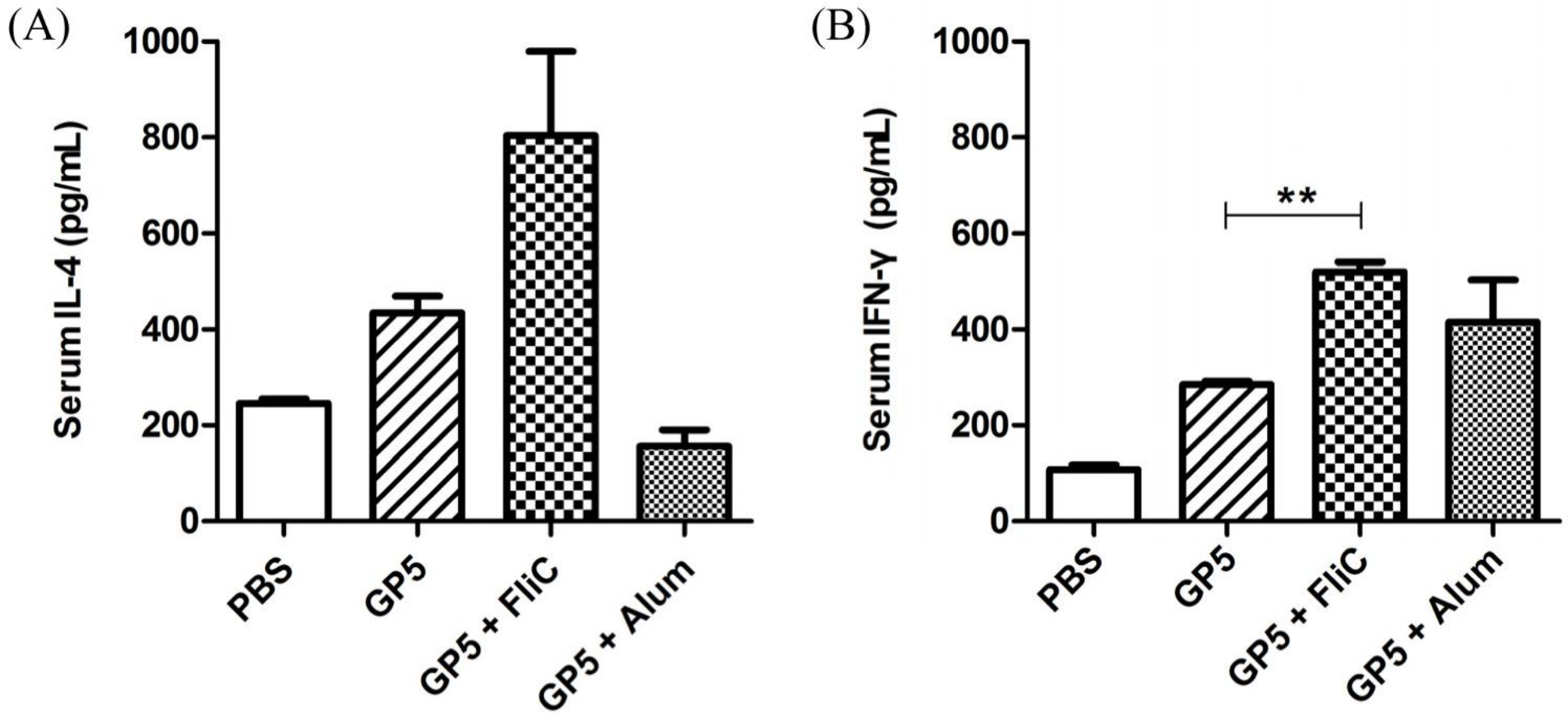
(A)
Serum IL-4 (pg/mL)
1000
800
600
400
200
0
PBS
GP5
GP5 + FliC
GP5 + Alum
(B)
Serum IFN-γ (pg/mL)
1000
800
600
400
200
0
**
PBS
GP5
GP5 + FliC
GP5 + Alum

1 **Supplementary Table 1.** PCR primers used in this study.

Primer name	Primer sequence (5′→3′)	Application
GP5-ORF F	ACTTAAGCTTGGTACCATGG	Amplification of GP5 ORF
GP5-ORF R	CGCTAGAGCGCTGGCAAGTG	Amplification of GP5 ORF
GP5a F	CCGGAATTCAACGCCAGCGACAACAAC	Amplification of GP5a fragment
GP5a R	**CACCGCCGCTTCCACCGCCACC**CTCCACTG CCCAGTCAAA	Amplification of GP5a fragment
GP5b F	**GTGGAAGCGGCGGTGGCGGAAGC**TGCATG TCCTGGCGCTA	Amplification of GP5b fragment
GP5b R	CCGCTCGAGCTAGAGACGACCCCATAGTTCCG CT	Amplification of GP5b fragment
GAPDH F	GCCTTCCGTGTTCCTACCC	Quantitative real-time PCR
GAPDH R	TGCCTGCTTCACCACCTTC	Quantitative real-time PCR
TLR5 F	TCCTCGTCATCACCCTTG	Quantitative real-time PCR
TLR5 R	AGCATTCTGTGCCCATTCA	Quantitative real-time PCR
IL-6 F	TACCACTCCCAACAGACCTG	Quantitative real-time PCR
IL-6 R	GTGCATCATCGTTGTTCATAC	Quantitative real-time PCR
TNF-α F	CATCTTCTCAAAATTCGAGTGACAA	Quantitative real-time PCR
TNF-α R	TGGGAGTAGACAAGGTACAACCC	Quantitative real-time PCR
IL-1β F	GAAATGCCACCTTTTGACAG	Quantitative real-time PCR
IL-1β R	GAGATTTGAAGCTGGATGC	Quantitative real-time PCR
IL-12 F	TTATGTTGTAGAGGTGGACTG	Quantitative real-time PCR
IL-12 R	GAAACTCTTTGACAGTGATGG	Quantitative real-time PCR
IL-10 F	CAATAACTGCACCCACTTCC	Quantitative real-time PCR
IL-10 R	CGGTTAGCAGTATGTTGTCCA	Quantitative real-time PCR

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