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

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

28 *3. Expression and purification of PRRSV GP5*

29 The GP5ab fragment was inserted into the *Eco*RI and *Xho*I digested expression vector
30 pET-32a or pGEX-6p-1 to create pET32a-GP5ab or pGEX-6p-1-GP5ab respectly. Recombinant
31 plasmid pET32a-GP5ab or pGEX-6p-1-GP5ab was purified with a plasmid purification kit
32 (TaKaRa, China) and transformed into host cells, *Escherichia coli (E. coli)*, BL21(DE3) pLysS.
33 Positive clones were identified on Luria-Bertani (LB) agar plates containing ampicillin (100
34 μg/ml). Protein expression was optimized according to a recent reference with minor
35 modification [Liu et al., 2009]. Briefly, the *E. coli* harboring pET32a-GP5ab or
36 pGEX-6p-1-GP5ab was cultured in LB liquid medium at 37°C with shaking until the optical
37 density (OD) of the culture at 600 nm reached 0.6. Then, isopropyl-β-dthiogalactoside (IPTG)
38 was added to a final concentration of 0.5 mM to induce expression at 25°C for 5 h. The empty
39 vector transformed bacteria were used as control. The bacteria were pelleted at 10,000 rpm, at
40 4°C for 10 min and re-suspended in TE buffer (50 mM Tris and 1 mM EDTA, pH8.0). Then, they
41 were digested with lysozyme at a final concentration of 100 μg/L at room temperature for 30 min.
42 The cell suspension was sonicated on ice for 30 min. Then the lysate was centrifuged at 10,000
43 rpm for 10 min at 4°C. The supernatant and the pellets were mixed with sodium dodecyl sulfate

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

48 *4. Purification of Salmonella typhimurium FliC flagellin*

49 Native FliC was purified from the attenuated *S.* Typhimurium SL7207 strain expressing FliC
50 according to a reference with minor modification [Ibrahim et al., 1985]. Briefly, bacteria were
51 grown in LB overnight at 37°C under aeration (80 rpm). Cells were washed once with
52 phosphate-buffered saline (PBS) and then adjusted to pH 2.0 with 1 M HCl and maintained at that
53 pH under constant stirring for 30 min at room temperature. The cell suspensions were centrifuged
54 to remove the cells and the pH of the supernatant was adjusted to 7.2 with 1 M NaOH. The
55 flagellar filaments were collected from the supernatant following ammonium sulfate precipitation.
56 The precipitate, which contained polymerized flagellin, was dissolved in approximately 5 ml of
57 distilled water and then transferred to dialysis tubing which had a molecular weight cutoff of
58 50,000 (Spectrum Medical Industries, USA). The protein content of FliC was determined with the
59 Bradford assay and by SDS-PAGE analyses.

60 *5. Western blotting*

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

65 (1:1000 diluted in PBS-0.05% Tween 20, PBST) at 37°C for 2 h. After washing three times with
66 PBST, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary
67 antibody (1:5000 diluted in PBST, Boster, China) at 37°C for 1 h. The protein bands were
68 visualized via diaminobenzidine enzyme-based color development in the dark and terminated by
69 distilled water.

70 *6. Endotoxin removal of GP5 and FliC*

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

76 *7. Administration of flagellin to conscious mice*

77 6-week-old female C57BL/6 mice were purchased from Comparative Medicine Centre of
78 Yangzhou University. They were housed in isolators and fed with a pathogen-free diet and water.
79 The procedures described in this study were approved by the Committee on the Ethics of Animal
80 Experiments of Yangzhou University, Yangzhou, China. C57BL/6 mice were injected
81 intraperitoneally with prepared endotoxin-free FliC, given at a dose of 2 μg/mouse. At selected
82 time-points (0 h, 1 h, 2 h, 6 h and 12 h) post injection, 5 mice were sacrificed by pentobarbital
83 overdose each time, and the spleen was removed for subsequent mRNA detection of TLR5 and
84 inflammatory cytokines.

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

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

101 *9. Mice immunization*

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

108 were tested by ELISA for antibodies and cytokines.

109 *10. Determination of anti-GP5 antibodies*

110 Serum IgG titers to GP5 were measured by ELISA according to a reference with minor
111 modification [Cui et al., 2012]. Briefly, 96-well microtiter plates were coated with the recombinant
112 GST-GP5 (0.5 ug/ml) in 50 mM carbonate buffer (pH 9.6) at 4°C overnight and blocked for 2 h at
113 37°C with 1% BSA in PBST. After washing three times with PBST, sera were added in a dilution
114 of 1:100 with a two-fold dilution series in the blocking buffer and incubated for 2 h at 37°C. After
115 five washes with PBST, Antigen specific antibodies were detected using goat anti-mouse IgG
116 conjugated to horse-radish peroxidase (1:10000 dilution) for 1 h at 37°C. The ELISA was
117 developed using TMB and H_2O_2 as substrates, and optical density was read at 450 nm (A450) with
118 an ELISA reader (Bio-TekEL 680, USA).

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

120 The levels of the cytokines (IL-4 and IFN-γ) in serum of each mouse were detected by
121 commercially available ELISA kits (BD Pharmingen, USA) according to the manufacturer's
122 protocol. Six standards (with expected concentrations of 1,000, 500, 250, 125, 62.5, 31.25 and
123 15.65 pg/ml) were prepared by serial dilution with an assay buffer kit, using a sensitivity
124 (threshold for detection) of 8.0 pg/ml. The prepared plates were analyzed using a microplate
125 reader at 450 nm.

126 *12. Statistical analysis*

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

129 version 5.0 (GraphPad Software, San Diego, CA). Statistical significance was determined at $P <$

130 0.05 (*), $P < 0.01$ (**) or $P < 0.001$ (***).

131 **References**

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151 ## Supplementary figure legends

152 **Supplementary Fig. 1. SDS-PAGE analysis of product expressed by recombinant bacteria of**

153 **BL21 (DE3)(pET32a-GP5) (left) and BL21(DE3)(pGEX-6p-1-GP5) (right).** The bacteria

154 harboring pET32a-GP5 or pGEX-6p-1-GP5 were induced with 0.5 mM IPTG at 25°C for 5 h, and

155 the bacterial protein were analyzed by SDS-PAGE respectively. (A) Lanes: M, molecular weight

156 markers; 1, Lysate supemaniant of BL21(DE3)(pET32a-GP5) induced by IPTG; 2, Inclusion

157 bodies of BL21(DE3)(pET32a-GP5) induced by IPTG; 3, product of BL21(DE3)(pET32a-GP5)

158 not induced; 4, product of BL21(DE3)(pET32a) induced by IPTG. (B) Lanes: M, molecular

159 weight markers; 1, product of BL21(DE3)(pGEX-6p-1) induced by IPTG; 2, Lysate supemaniant

160 of BL21(DE3)(pGEX-6p-1-GP5) induced by IPTG; 3, Inclusion bodies of

161 BL21(DE3)(pGEX-6p-1-GP5) induced by IPTG.

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164 **Supplementary Fig. 2. Levels of TLR5 (A), IL-6 (B), TNF-α (C), IL-1β (D), IL-12 (E) and**

165 **IL-10 (F) mRNAs in spleen of mice following injection with prepared endotoxin-free FliC.**

166 Female C57BL/6 mice were immunized intraperitoneally with prepared endotoxin-free FliC, given

167 at a dose of 2 μg/mouse. Data shown are the fold changes in mRNA expression compared with

168 that in 0 h controls, based on five mice for each time and determined by qRT-PCR. Error bars

169 indicate standard deviations of the means. Statistical significance was determined at $P < 0.05$ (*)

170 or $P < 0.001$ (***).

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

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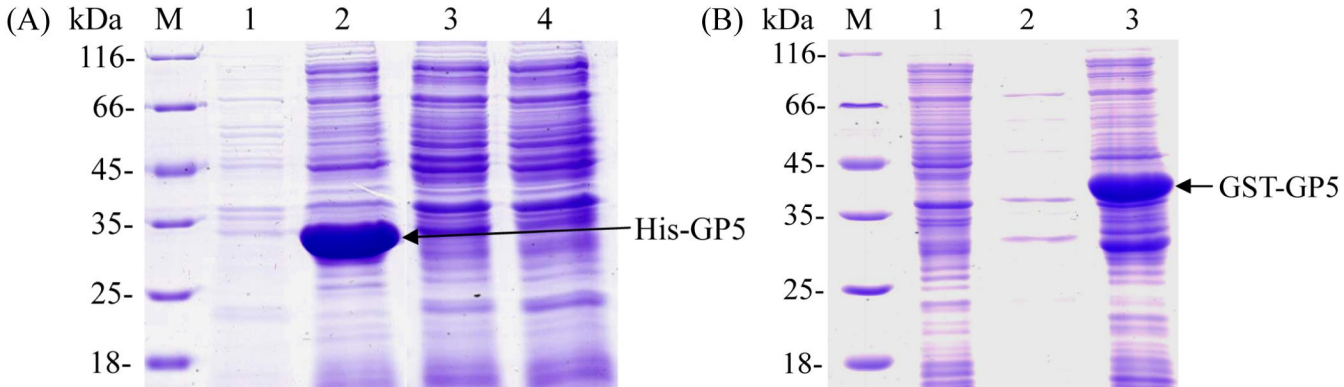
(A)
kDa
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4
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66-
45-
35-
25-
18-
His-GP5
(B)
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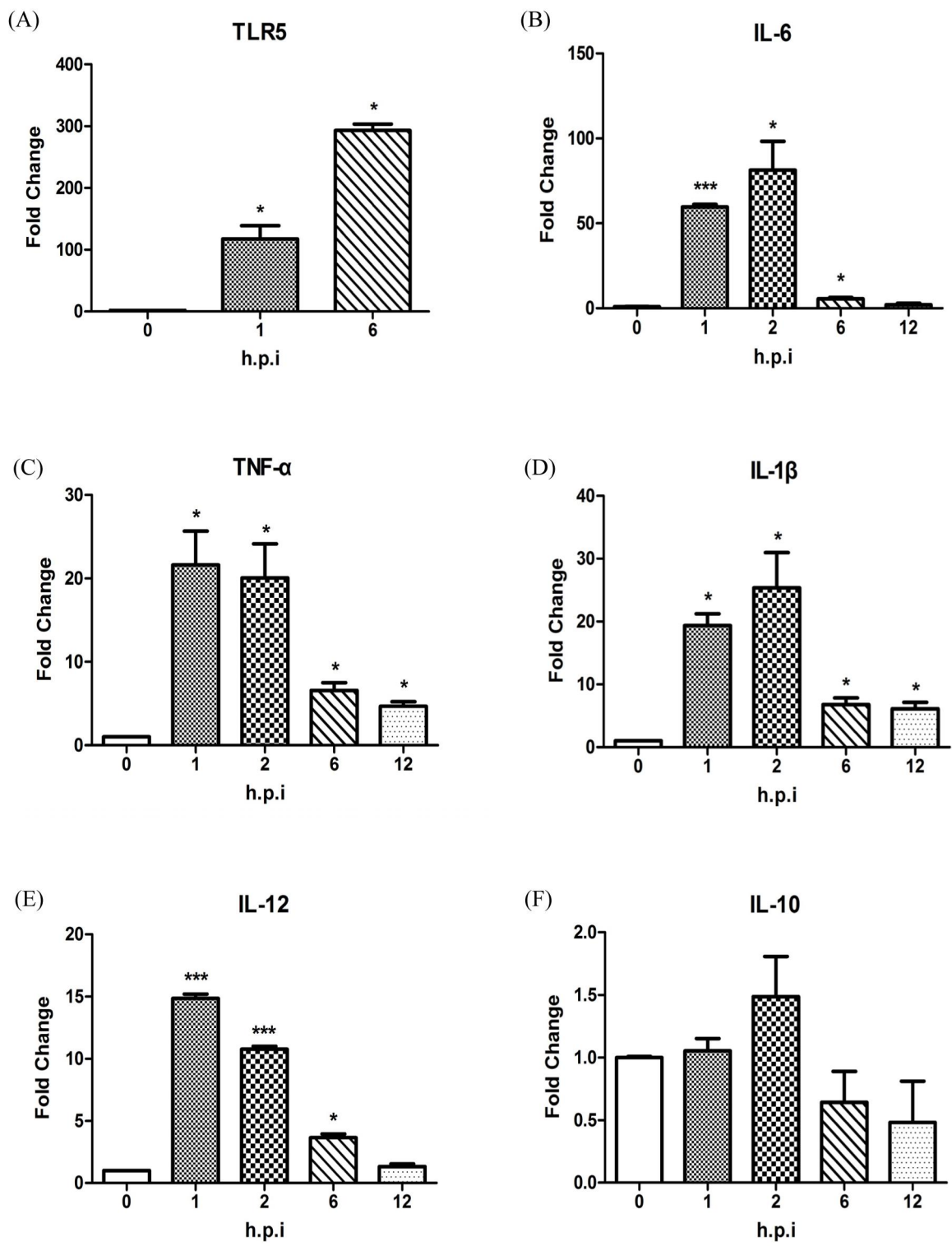
35-
25-
18-
GST-GP5


(A)
TLR5
Fold Change
400
300
200
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6
h.p.i
(B)
IL-6
Fold Change
150
100
50
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1
2
6
12
h.p.i
(C)
TNF-α
Fold Change
30
20
10
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2
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h.p.i
(D)
IL-1β
Fold Change
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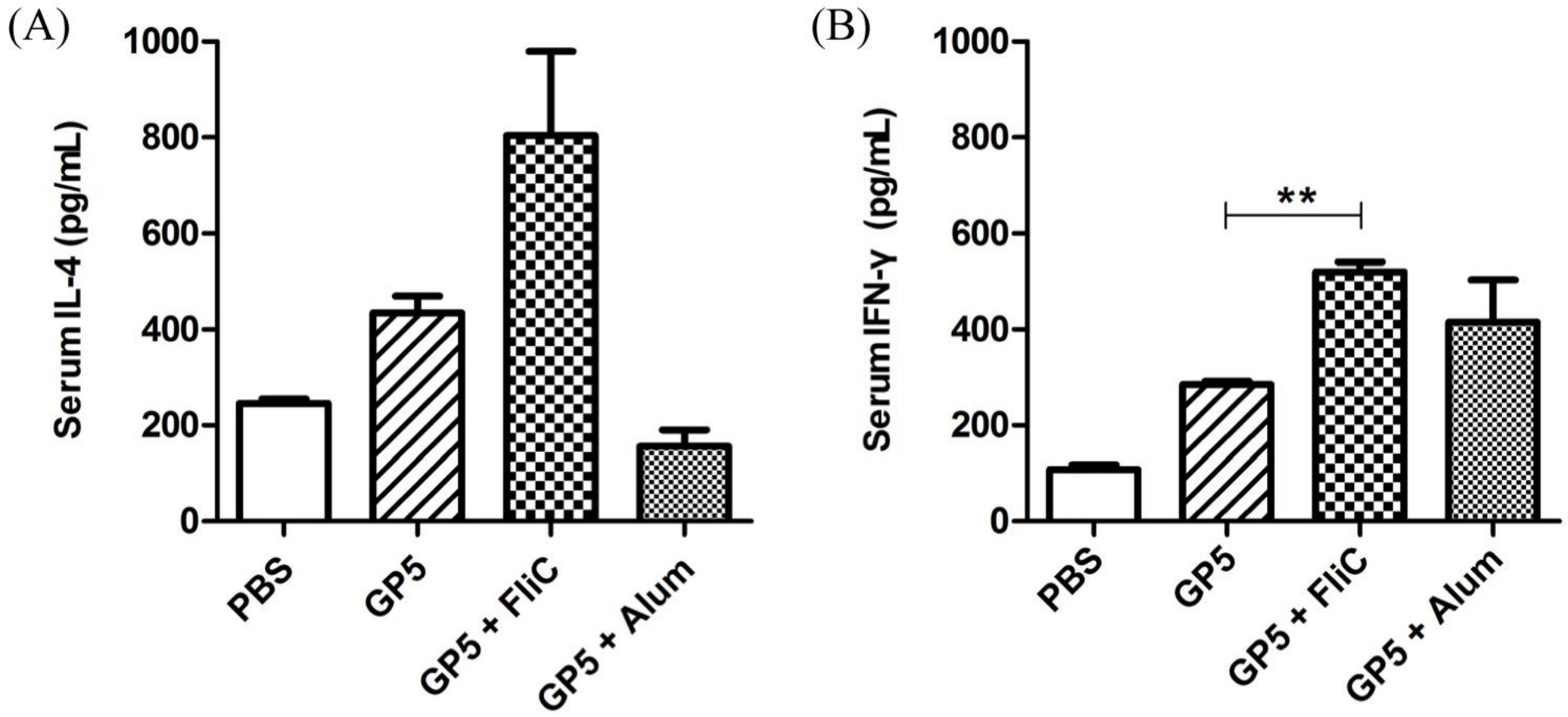
(A)
Serum IL-4 (pg/mL)
1000
800
600
400
200
0
PBS
GP5
GP5 + FliC
GP5 + Alum
(B)
Serum IFN-γ (pg/mL)
1000
800
600
400
200
0
**
PBS
GP5
GP5 + FliC
GP5 + Alum

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**CTCCACTGCCCAGTCAAA	Amplification of GP5a fragment
GP5b F	**GTGGAAGCGGCGGTGGCGGAAGC**TGCATGTCCTGGCGCTA	Amplification of GP5b fragment
GP5b R	CCGCTCGAGCTAGAGACGACCCCATAGTTCCGCT	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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