Effects of astragalus polysaccharide on the adhesion-related Immune response of endothelial cells stimulated with CSFV *in vitro*

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Background: Astragalus polysaccharide (APS) has immunomodulatory activities on porcine peripheral blood mononuclear cells. The immunomodulatory effects of APS on porcine endothelial cells (ECs) expose to classical swine fever virus (CSFV) remain unknown.

Methods: The virus was titrated using an indirect immune biotin enzyme standard method to confirm that porcine ECs were susceptible to CSFV infection and to determine the TCID50 of CSFV (C-strain). Porcine ECs were cultured with CSFV in the presence of APS. Relative quantitative PCR was used to assess the mRNA expression of factors that influence EC adhesion and immunity.

Results: The expression of adhesion factors mRNA increased following stimulation with CSFV; this effect was inhibited by pre-exposing the cells to APS. In addition, the expression of growth factors and some immune factors increased after infection with CSFV; this increase in tissue factor (TF), transforming growth factor (TGF-β), and interleukin-8 (IL-8) could be inhibited by the addition of APS. The immune response mediated by Toll-like receptor 4 (TLR4) in ECs may be unregulated by CSFV as it was also inhibited by pre-treatment with APS.

Discussion: The addition of APS to the culture can obviously regulate the expression of molecules related to the adhesion, growth, and immune response of ECs, as well as the production of cytokines. Therefore, it may have the potential to be an effective component in vaccines against CSFV.

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Abstract

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Results: The expression of adhesion factors mRNA increased following stimulation with CSFV; this effect was inhibited by pre-exposing the cells to APS. In addition, the expression of growth factors and some immune factors increased after infection with CSFV; this increase in tissue factor (TF), transforming growth factor (TGF-β), and interleukin-8 (IL-8) could be inhibited by the addition of APS. The immune response mediated by Toll-like receptor 4 (TLR4) in ECs may be unregulated by CSFV as it was also inhibited by pre-treatment with APS.

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Introduction

Classical swine fever (CSF) is a highly contagious disease affecting swine and leads to

tremendous economic losses worldwide. The virus of CSF (CSFV) is a member of the *Pestivirus* genus of the *Flaviviridae* family, which also includes viruses that cause bovine viral diarrhea/mucosal disease and border disease.[1]

A CSFV infection in pigs causes hemorrhagic disease characterized by disseminated intravascular coagulation, thrombocytopenia, and immunosuppression.[2] Many different cell types have been studied in investigations of CSFV including macrophages.[3, 4] Vascular endothelial cells (ECs) maintain a hemostatic balance by providing a quiescent, anti-thrombotic barrier.[5] ECs can be infected by CSFV and finally induces vascular diffuse lesions in the host.[6, 7] However, ECs can be rapidly activated by pathogens to express a proinflammatory and procoagulant phenotype to eliminate infection.[8] Evidence supports the notion that ECs play a critical role in the recruitment of immune cells, as well as influencing the outcome of the immune response.[9, 10, 11]

Some factors can modulate EC function, including vascular endothelial growth factor (VEGF), VEGF is a multifunctional cytokine that plays a critical role in the regulation of angiogenesis and is also a potent mitogen that specifically targets ECs.[12] .The activation of tissue factor (TF) may regulate the initiation of the coagulation protease cascade in various cellular settings[13] and is essential for triggering angiogenesis via VEGF production by human fibroblasts.[14]

Cell adhesion molecules (CAMs) including P-selectin and E-selectin, which are involved in cell tropism and pathogenesis during the course of viral infections.[15] The expression of tumor necrosis factor (TNF)-α may contribute to the leukopenia in pigs infected with CSFV by promoting apoptosis.[16] CSFV induces proinflammatory cytokines and tissue factor (TF) expression and inhibits apoptosis and interferon synthesis during the infection of porcine vascular ECs: The virus infection inhibited polyinosinic-polycytidylic acid [poly(I:C)]-induced α/β IFN

(type I IFN) synthesis and there was a 100-fold increased capacity to resist apoptosis induced by poly(I:C) when the porcine kidney cell line SK-6 was infected with CSFV.[10] Type I IFNs have a bifunctional role in limiting the spread of the virus and inducing an antiviral state in uninfected cells while promoting apoptosis in infected cells. Furthermore, virus-induced apoptosis can be inhibited by anti-IFN-α/β antibodies.[17]

Endothelial-to-mesenchymal transition (EMT) contributes to fibrosis, which is a major cause of human death and disability.[18, 19] The progression of fibrosis due to aortic banding is associated with the increased expression of mRNA encoding transforming growth factor-β1 (TGF-β1).[20]

Astragalus polysaccharide (APS), which have been identified as an acidic heteropolysaccharide mainly composed of glucose, galactose, arabinose, rhamnose and small amount of mannose, fructose, ribose, xylose, fucose, glucuronic acid, and galacturonic acid, is extracted from *Astragalus membranaceus* (AM) roots and generally purified by dissolving in the distilled water, dialysis and lyophilization.[21] APS is the main active ingredient in AM with molecular weights in the range of 8.7×10^3–4.8×10^7g mol^{-1}, [22] has a substantial effect on exerting immunomodulatory effects in macrophage cells,[23] activating the immune system by clearing immune complexes,[24] enhancing the transformation of T lymphocytes, as well as activating B lymphocytes and dendritic cells (DC).[25, 26] Moreover, APS has been used as an immunomodulator for the vaccination of pigs and to enhance the protective efficacy against foot-and-mouth disease virus (FMDV).[27] Orally administered APS has also been shown to significantly enhance the efficacy of FMDV vaccination and has important implications for the further use of APS as a novel adjuvant.[28] Moreover, it was reported that AM markedly attenuated the inhibition of vasorelaxation and downregulation of cGMP levels in ECs, and APS exhibited a

tendency to reverse both depressive responses.[29] Furthermore, APS plays a role in attenuating the infiltration of inflammatory cells (e.g., neutrophils and monocytes) by decreasing the expression of adhesion molecules, the release of chemokines, and induction of enzymes.[30] Some of the genes altered by APS treatment are associated with the activation of the immune proinflammatory response, such as the promotion of interleukin-1, 8, and interferon-γ production. Thus, APS serves to improve immune defense functions while resisting the invasion of external pathogens.[31]

In our previous study, we found that APS exhibited immunomodulatory effects on cells exposed to CSFV and had the potential to be an effective adjuvant in vaccines against CSFV.[32] In the present study, we evaluated the effect of APS on the cellular responses following a CSFV with the aim of identifying a potential method of combating this hemorrhagic infection.

Materials and Methods

Astragalus polysaccharides and virus

APS (Freeze-dried) was kindly provided by Dr. Fenghua Liu (Beijing University of Agriculture, Beijing, China) and stored at room temperature. A rabbit propagated C-strain of CSFV was kindly provided by Dr. Hongwu Lang (China Institute of Veterinary Drug Control, Beijing, China) and stored at -20°C.

Virus titration by an indirect immune biotin enzyme standard method

Porcine hip artery endothelial cells (ECs) were purchased from the China Institute of Veterinary Drug Control in Beijing. ECs (1×10^5 /mL) were incubated in M199 culture medium (Invitrogen, Carlsbad, CA, USA) containing 2 mM L-glutamine enriched with 100 U/ml penicillin/streptomycin (Sigma, St. Louis, MO) and 10% heat-inactivated fetal calf serum. These were incubated in a 96-well microplate (160 μL/well) for 24 h (5% CO_2, 37°C). The CSFV stock was diluted 10-fold × 10 (from 5 mg/mL to 5×10^{-10} mg/mL), and added to the microplate at 40 μL/well, with each concentration occupying eight wells. After 48 h incubation (5% CO_2, 37°C), the culture medium was aspirated and the plate was washed with PBS.

Hyperimmune rabbit-anti-CSFV serum (China Institute of Veterinary Drug Control, Beijing) was inactivated at 56°C for 30 min before adding it to the culture plate (1:400) at 37°C. The plates were washed with PBS three times after 1 h. Biotin-labeled sheep anti-swine antibodies (China Institute of Veterinary Drug Control, Beijing) were added to the culture plate (20μg/mL, 100μL/well) at 37°C. Horseradish peroxidase-labeled avidin-biotin-peroxide complex (ABC) (5mg/mL) was added (100μL/well) after 1 h. The culture plates were washed with PBS three times after 30 min. 3,3N-Diaminobenzidine Tetrahydrochloride (China Institute of Veterinary Drug Control, Beijing) was added (50μL/well) to develop the color.

114 **Cell cultures**

115 ECs were cultured in M199 culture medium in 25 cm^2 culture dishes. A final concentration of
116 10^3 $TCID_{50}$/mL CSFV or 10 μg/mL APS was added after 48 h subculturing. Cells were allocated
117 to one of six groups (each group had three replicates): (1) Medium (cells cultured only in
118 medium); (2) APS pre-treatment (APS added 24 h after subculturing); (3) APS (APS added 48 h
119 after subculturing); (4) CSFV (CSFV added 48 h after subculturing); (5) CSFV + APS pre-
120 treatment (APS added 24 h and CSFV added 48 h after subculturing); (6) CSFV + APS (CSFV
121 and APS both added 48 h after subculturing). The cells and supernatants in each of the six groups
122 were harvested at 3 h and 6 h after the addition of CSFV (48 h after subculturing).

123 **RNA isolation and reverse transcription**

124 Trizol reagent (Invitrogen, Carlsbad, CA, USA) was used to extract the total RNA from the
125 ECs, and the extracted RNA was dissolved in RNase-free water (Qiagen, Valencia, CA, USA).
126 The integrity of each RNA sample was ascertained by agarose gel electrophoresis. The RNA
127 purity (OD260/OD280 absorption ratio > 1.9) and its quantity were determined and verified using
128 a NanoDrop® ND-2000C Spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE,
129 USA).

130 The reverse transcription (RT) reaction was initiated by adding 2 μg of total RNA and 0.5 μg
131 of oligo (dT_{15}) to 12.5 μL of sterile, distilled water and heated to 72°C for 5 min. After cooling
132 the samples on ice, 10 mM dithiothreitol (DTT), 0.5 mM of each dNTP, 5 × first strand buffer
133 and 200 U Superscript II RNase H–Reverse Transcriptase (Promega, Madison, WI, USA) was
134 added. The mixture was stabilized at 25°C for 10 min and subsequently incubated at 42°C for 60

135 min for the RT reaction. Thereafter, the temperature was raised to 70°C for 15 min to inactivate
136 the reverse transcriptase. The synthesized cDNA was stored at –20°C until use.

137 **Quantitative real-time PCR**

138 Quantitative real-time PCR was performed in a 20 μL PCR reaction containing a final
139 concentration of 10 μL 2 × SYBR Premix DimerEraser and 0.4 μL ROX (passive reference dye)
140 (Promega, Madison, WI, USA), 2 μL of cDNA of each sample, and 0.5 μM each of the forward
141 and reverse primers. The thermal cycling profile consisted of four steps: (1) denaturation at 95°C
142 for 30 s; (2) amplification for 45 cycles of denaturation at 95°C for 5 s, annealing at 55°C for 30
143 s, and extension at 72°C for 1 min; (3) melting curve by 95°C for 15 s, 60°C for 1 min, and 95°C
144 for 30 s; and (4) cooling at 4°C. All reactions were conducted in triplicate using a 7500 Real-
145 Time PCR System (Applied Biosystems, Foster City, CA, USA). A non-template nuclease-free
146 water control was included for each run. The primers (Applied Biosystems, Foster City, CA,
147 USA) for P-selectin, E-selectin, VEGF, TF, TLR4, IFN-α, β, γ, IL-1, 6, 8, and hypoxanthine
148 phosphoribosyl-transferase (HPRT) are listed in Table 1. SyBgreen Gene Expression Assays
149 (Promega, Madison, WI, USA) were used in our study. To evaluate the relative quantification of
150 cDNA, the cycle threshold (Ct) value of the target genes for each sample were determined and
151 normalized to the Ct-values of the housekeeping gene, HPRT. The results were presented as the
152 fold change using the $2^{-\Delta\Delta Ct}$ method.

153 **Protein quantification by ELISA**

154 The concentration of IFN-α, IFN-γ, and TGF-β in the cell culture supernatants was assayed
155 according to the manufacturer instructions of commercial ELISA kits (R&D Systems,
156 Minneapolis, MN, USA).

157 **Statistical analysis**

158 SPSS software was used for all statistical evaluations (SPSS Version 16.0, SPSS Inc.,
159 Chicago, Illinois, IL, USA). A one-way analysis of variance (ANOVA) was used to test for
160 differences between the groups, and the data were analyzed via a non-parametric Dunnett's test.
161 The results are presented as the means ± SEM. *P*-values less than 0.05 were considered to be
162 statistically significant.

163 ## Results

164 ## $TCID_{50}$ of CSFV

165 Puce cytoplasm reveals which ECs were infected with CSFV (Figure 1). The results are
166 expressed as 50% of the tissue culture infective dose ($TCID_{50}$): 10^3 $TCID_{50}$/mL is equivalent to
167 10 μg/mL of the CSFV C-strain.

168 **mRNA expression of E-selectin, P-selectin, VEGF, TF, TLR-4, IFN-α, IFN-β and IL-1, 6,**
169 **and 8 on ECs following infection with CSFV in the presence or absence of APS**

170 Compared with medium alone (Figure 2A), the mRNA expression of E-selectin was
171 significantly increased 3 h after stimulation with CSFV in Group 4 ($P = 0.043$) and was even
172 higher in Group 6 ($P < 0.001$). After 6 h of stimulation, the mRNA expression of E-selectin

173 decreased in Group 3 ($P < 0.001$) but increased in Group 5 ($P < 0.010$).

174 After 3 h of stimulation with CSFV (Figure 2B), a significant decrease in the mRNA
175 expression of P-selectin ($P = 0.001$) compared to medium alone was detected in Groups 2 and 3,
176 which were treated with APS. At 6 h post-stimulation, the mRNA expression of P-selectin was
177 significantly increased in the groups stimulated with CSFV (Groups 4, 5, and 6) compared with
178 groups that were not stimulated with CSFV (Groups 1, 2, and 3) ($P < 0.010$). The expression of
179 P-selectin was lower after stimulation with CSFV plus pretreatment with APS compared with the
180 group stimulated with CSFV alone ($P < 0.001$), whereas the expression was higher after
181 stimulation with both CSFV and APS simultaneously ($P < 0.001$).

182 VEGF mRNA expression in the ECs (Figure 2C) was higher in the presence of APS ($P =$
183 0.010) or CSFV ($P = 0.025$) alone but decreased in the presence of both CSFV and APS
184 compared to the medium alone at 3 h post-stimulation. Moreover, the mRNA expression of
185 VEGF was higher following stimulation with CSFV after pretreatment with APS compared to
186 all the other test groups ($P = 0.001$). After 6 h of stimulation, VEGF mRNA expression was
187 significantly increased in all stimulated groups compared to the medium alone ($P < 0.001$).
188 VEGF mRNA expression was higher in the presence of CSFV plus APS (Groups 5 and 6) than
189 in the presence of CSFV alone ($P < 0.050$). In addition, the expression of VEGF mRNA was
190 higher in the presence of CSFV following pretreatment with APS than in the group
191 simultaneously treated with CSFV and APS ($P < 0.001$).

192 After 3 h of stimulation, the expression of TF mRNA (Figure 2D) was higher in the
193 presence of APS alone compared to APS pre-treatment alone ($P = 0.005$); TF mRNA expression
194 in the ECs was significantly increased in the presence of CSFV ($P \leq 0.010$). After 6 h of

195 stimulation, TF expression was higher following APS pre-treatment alone than in the presence
196 of APS alone (P = 0.018). TF mRNA expression was increased in the presence of CSFV alone
197 ($P = 0.001$) but not following CSFV plus APS.

198 TLR4 mRNA expression (Figure 3A) in the ECs was significantly increased in the presence
199 of CSFV ($P < 0.010$) but not in the presence of CSFV plus APS pre-treatment after 3 h of
200 stimulation. However, TLR4 mRNA expression in the ECs was significantly increased in the
201 presence of CSFV plus APS when administered simultaneously (Group 6) ($P < 0.010$), which
202 was higher than in the presence of CSFV alone ($P = 0.016$). At 6 h post-stimulation, the mRNA
203 expression of TLR4 was significantly increased in the presence of APS alone ($P < 0.001$)
204 compared to the medium controls.

205 At 3 h post-stimulation, the expression of IFN-α mRNA increased (Figure 3B) in the
206 presence of APS alone ($P = 0.050$) and CSFV alone ($P = 0.006$). At 6 h post-stimulation, the
207 mRNA expression of IFN-α was increased in Groups 2 and 5 ($P < 0.001$). At 3 h after
208 stimulation, IFN-β mRNA expression (Figure 3C) was lower in the presence of APS ($P = 0.026$)
209 or CSFV alone ($P = 0.016$) than in medium control group; however, it was higher in the presence
210 of CSFV pretreated with APS ($P = 0.050$). At 6 h after stimulation, IFN-β mRNA was decreased
211 in the majority of the groups except for Group 2 compared to the medium alone.

212 At 3 h after stimulation, the mRNA expression of IL-1 (Figure 3 D) was increased in Group
213 3 ($P = 0.012$), 4 ($P = 0.005$), 5 ($P = 0.001$), and 6 ($P = 0.009$). At 6 h post-stimulation, IL-1
214 mRNA expression was only increased in the presence of APS alone ($P < 0.001$).

215 Compared to the medium control at 3 h after stimulation, IL-6 mRNA expression (Figure 3E)
216 was decreased in the ECs pretreated with APS ($P = 0.026$), while it increased in the presence of

APS alone (P = 0.033). At 6 h after stimulation, IL-6 mRNA expression was increased in Groups 2 and 3, in which APS was added at different times (P < 0.050). IL-6 mRNA expression was significantly increased in the presence of CSFV pretreated with APS (P = 0.008).

At 3 h after stimulation, the expression of IL-8 mRNA (Figure 3F) was significantly increased in Groups 3 and 4 (P = 0.002) and was increased in the group simultaneously treated with CSFV plus APS (Groups 5 and 6) (P = 0.001). At 6 h after stimulation, IL-8 mRNA expression was significantly increased in Groups 3 (P = 0.001) and 4 (P = 0.030). IL-8 mRNA expression was much lower in the presence of CSFV plus APS (Group 6) than in the presence of CSFV alone (P = 0.043).

Cytokine production following CSFV is modulated in ECs treated with APS

IFN-α expression in the EC supernatants was increased in all the stimulated groups (Groups 2, 3, 4, 5 and 6; Figure 4A); particularly following stimulation with APS (Groups 2 and 3). The expression of IFN-α was significantly increased after stimulation with CSFV but was lower in the presence of CSFV plus APS (Groups 5 and 6). Moreover, IFN-α expression was lower in the presence simultaneously of CSFV plus APS than in the presence of CSFV following pre-treatment with APS.

IFN-γ expression in the EC supernatants was increased in the presence of APS alone but decreased significantly in the presence of CSFV (Groups 4, 5 and 6; Figure 4B). In addition, IFN-γ expression was much lower following stimulation with CSFV plus APS (Groups 5 and 6) compared to the presence of CSFV alone and was the lowest in the presence of CSFV following pre-treatment with APS.

239 TGF-β expression (Figure 4C) in the EC culture supernatants was increased in the presence
240 of APS alone, CSFV alone, and CSFV plus APS, but decreased in the presence of CSFV
241 following pretreatment with APS.

242

243 ## Discussion

244 CSFV infection induced a functional decrease in the pro-coagulant activity of the ECs, which
245 were highly susceptible to CSFV infection.[33] the inhibitory effects of APS on porcine viruses
246 infection have verified [34] and APS has been observed to regulate the levels of immunomodulating
247 compounds in many different cell types.[35, 36] The manifestation of adhesion molecules, including
248 P-selectin and E-selectin, was inhibited by APS and persisted while the ECs were stimulated with
249 CSFV, which could promote the expression of both P- and E-selectin (Figure 2AB). Similarly, the
250 increase of TF (Figure 2D), TGF-β (Figure 4C), and IL-8 (Figure 3F) production in response to
251 CSFV was inhibited by APS.

252 The coordinated expression of adhesion molecules leads to the localization of leukocytes to a
253 tissue or organ, and members of the selectin family initially mediate the transition from rapidly
254 flowing the bloodstream to rolling along the endothelium.[37] E-selectin is an early mediator of
255 leukocyte-endothelial adhesion and is expressed on activated endothelium.[38] The data presented
256 in the present study unequivocally demonstrated that APS inhibited the activation of E-selectin
257 caused by CSFV which was also delayed by APS pre-treatment, indicating that APS has a
258 preventive effect on the hemorrhaging stimulated by CSFV. However, the addition of APS
259 following infection with CSFV induced the co-stimulation of E-selectin which fade away after 3

hours, indicating that the addition of APS may enhance vaccine effectiveness, but can't maintain for a long time (Figure 2A).

Our results also revealed that VEGF expression was increased by CSFV and could be further enhanced with the pre-treatment of APS (Figure 2C). This finding indicates that CSFV and APS may elicit an angiogenic response via VEGF.[10, 39] Furthermore, the release of TF may be important in mediating the activation of both coagulation and fibrinolytic mechanisms.[40] We found that APS inhibited the expression of TF, which was upregulated by CSFV. The inhibition of APS appeared later than upregulation of CSFV (Figure 2D). This phenomenon involved in disseminated intravascular coagulation and thrombocytopenia during the progression of CSFV.[2]

As a pattern recognition receptor, TLR4 can activate the innate immune response to both bacterial and viral pathogens, including RNA viruses.[41] We demonstrated that the immune response mediated by TLR4 in ECs was induced by CSFV, which contributes to the ability of CSFV to replicate without exerting a cytopathic effect (CPE).[42, 43] We found that APS can inhibit the expression of TLR4 in ECs following infection with CSFV (Figure 3A) at a later time, while co-stimulate and promote it at an early time point, indicating that APS can regulate innate immunity and vessel remodeling via TLR4 expression which has been proved in macrophages lately.[23]

The suppression of IFN-β plays a role in the inability of porcine viruses to spread and cause disease in susceptible animals;[44] however, this cytokine was upregulated when the ECs were pretreated with APS (Figure 3C). Our results demonstrate that CSFV inhibited the expression of IFN-γ (which always fails to be produced in cells infected with CSFV [42]). IFN-γ profoundly inhibited African swine fever virus replication in porcine macrophages,[45] as well as the cytopathic

effect produced by CSFV;[46] however, this inhibition remained unchanged following the addition of APS (Figure 4B).

IL-1, IL-6, and IL-8 are typical examples of the multifunctional cytokines involved in the regulation of the immune response, hematopoiesis, and inflammation.[47, 48] Following an infection with CSFV, there is an initial and brief increase in the transcript levels of IL-1 and 8,[42] which was confirmed in our study (Figure 3DEF); the increase in IL-8 was inhibited by APS.

In conclusion, the data from the present study indicate that APS induced the production of immune factors and prevented an increase in adhesion molecules, which were inhibited and activated by CSFV, respectively. In some cases, the addition of APS induced the co-stimulation of factors associated with CSFV, indicating that the addition of APS may enhance vaccine efficacy. Thus, APS may have an modulation effect on ECs exposed to CSFV and utilize different mechanisms to influence the adhesion-related immune response of ECs during the hemorrhagic treatment. However, *in vitro* data is not sufficient to conclude that APS has a preventive effect on the hemorrhaging induced by CSFV. These possibilities warrant further study to evaluate whether APS may be an effective adjuvant in *in vivo* clinical vaccine trials against CSFV.

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Figure Legends

Figure 1. ECs infected with CSFV titrated by indirect immunofluorescence. The deep brown in the cells indicates which parts were infected with CSFV.

Figure 2. The relative mRNA expression of P-selectin (A), E-selectin (B), VEGF (C) and TF (D) in porcine ECs. The relative mRNA expression in porcine ECs cultured with either medium alone (Medium), pretreated with APS (APS pre-treated), APS, CSFV, CSFV following pretreatment with APS (CSFV+APS pre-treated), or CSFV plus APS (CSFV+APS). Different texture filled in bars shows different culture conditions which illustrated in A. Gene expression was analyzed by SYBE Green RT-PCR. Data are presented as the means ± SEM of three independent experiments. Within the same time point: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Figure 3. Relative mRNA expression of TLR4 (A), IFN-α (B), IFN-β (C), IL-1 (D), IL-6 (E) and IL-8 (F) in porcine ECs. Relative mRNA expression in porcine ECs cultured with either medium alone (Medium), pretreatment with APS (APS pre-treated), APS, CSFV, CSFV following pretreatment with APS (CSFV+APS pre-treated), or CSFV plus APS (CSFV+APS). Different texture filled in bars shows different culture conditions which illustrated in A. Gene expression was analyzed by SYBE Green RT-PCR. Data are presented as the mean ± SEM of three independent experiments. Within the same time point: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Figure 4. Enzyme-linked immunosorbent assay of IFN-α, IFN-γ and TGF-β in cell culture supernatants. Porcine ECs were cultured with either medium alone (Medium), pretreated with APS (APS pre-treated), APS, CSFV, CSFV following pretreatment with APS (CSFV+APS pre-treated), or CSFV plus APS (CSFV+APS). Data are presented as the means ± SEM of three

441 independent experiments. Comparisons between all groups $P < 0.001$.

442

443 Table 1. Sequences of the oligonucleotide primers used for real-time RT-PCR.

444

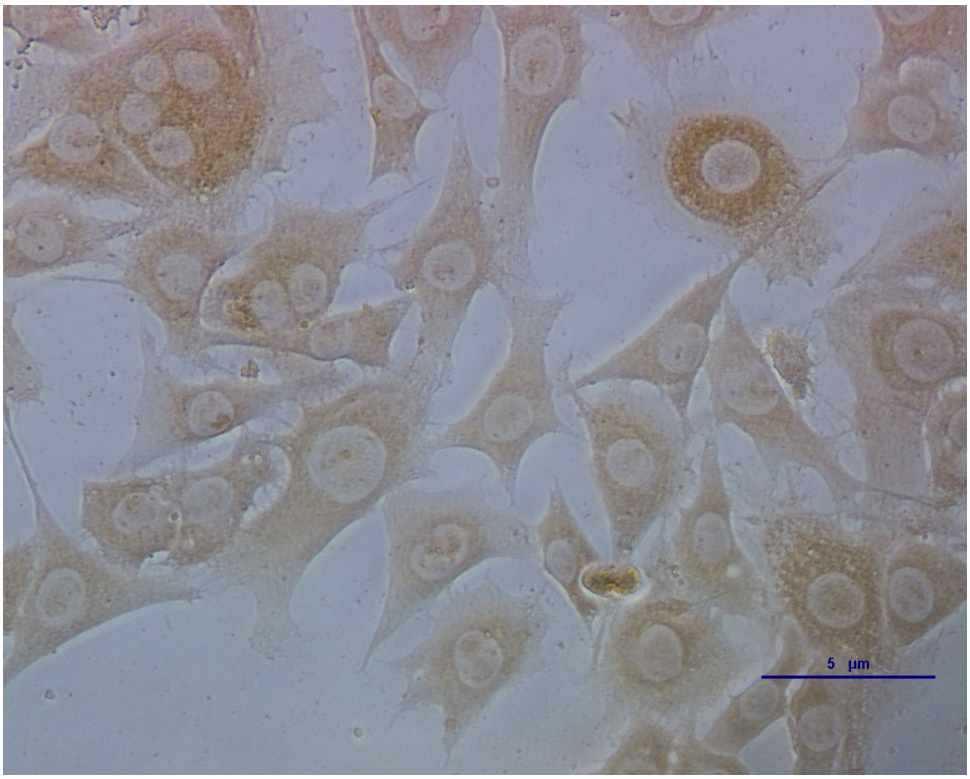
Gene specificity		Oligonucleotide sequences (5'-3') of primers
HPRT[a]	F	GTGATAGATCCATTCCTATGACTGTAGA
	R	TGAGAGATCATCTCCACCAATTACTT
P-selectin[b]	F	AACGGAGGGGAGGCAACAAGAC
	R	GTGAGGGGACCAAGAGAAG
E-selectin[b]	F	ATGATTGCTTCACAGTTTCTCT
	R	TCACATGTCACAGCTTTACACG
TLR-4[c]	F	AAGGTTATTGTCGTGGTGT
	R	CTGCTGAGAAGGCGATAC
VEGF[d]	F	ATGAACTTTCTGCTGTCTTGGGTG
	R	TCACCGCCTCGGCTTGTCACATCT
TF[d]	F	TTCAAGAC（A/C）ATT（T/C）TGGAGTGG
	R	AGGGGGAGTTGGTAAAC
IL-1[d]	F	ACTTCCTGGGGACGGCATGGATAAA
	R	GCATCATTCAGGATGCACTGGTGGT
IL-6[d]	F	GCTGCTTCTGGTGATGGCTACTGCC
	R	TGAAACTCCACAAGACCGGTGGTGA
IL-8[d]	F	AGCCCGTGTCAACATGACTTCC
	R	GAATTGTGTTGGCATCTTTACTGA
IFN-α[d]	F	ATGGCCCCAACCTCAGCCTTC
	R	TCACTCCTTCTTCCTGAGTCT
IFN-β[d]	F	ATGGCTAACAAGTGCATCCTCCAA
	R	TCAGTTCCGGAGGTAATCTGTAAG

445

446 HPRT, hypoxanthine phosphoribosyl-transferase; IFN-α, interferon α; IL-1, interleukin 1; F, forward primer; R, reverse primer.

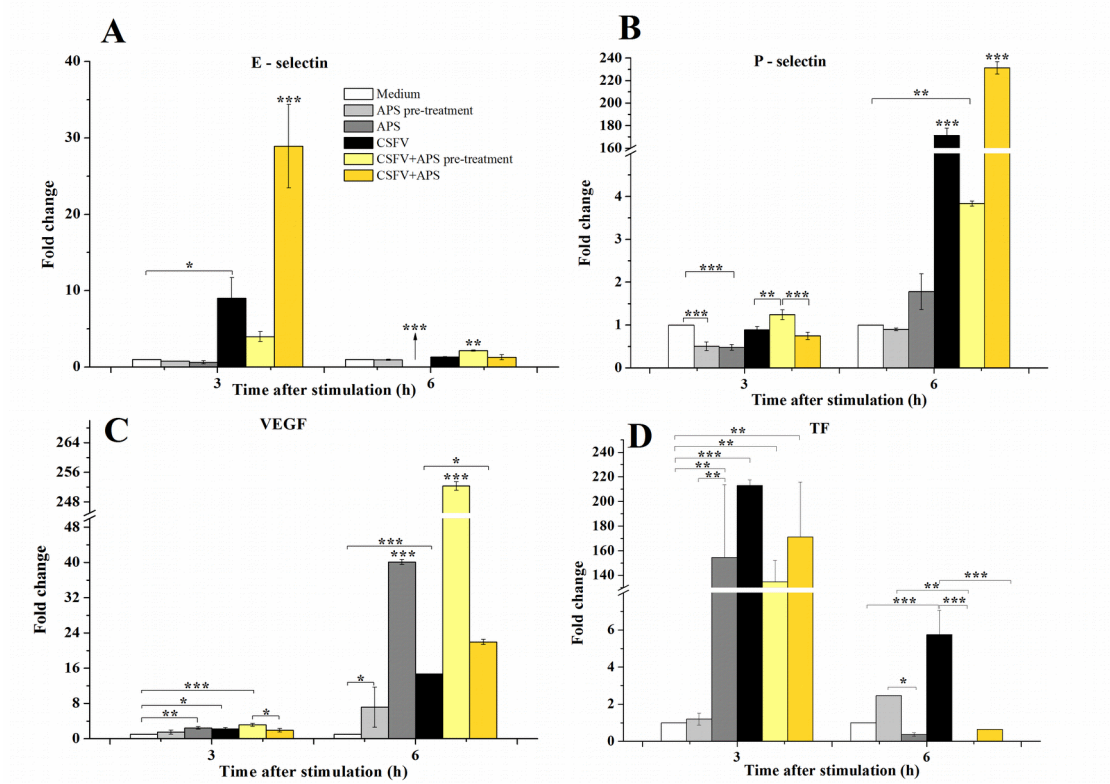
447 a; [49] b; [50] c; [51] d. [42]

448 **Figure 1**



449

450 **Figure 2**



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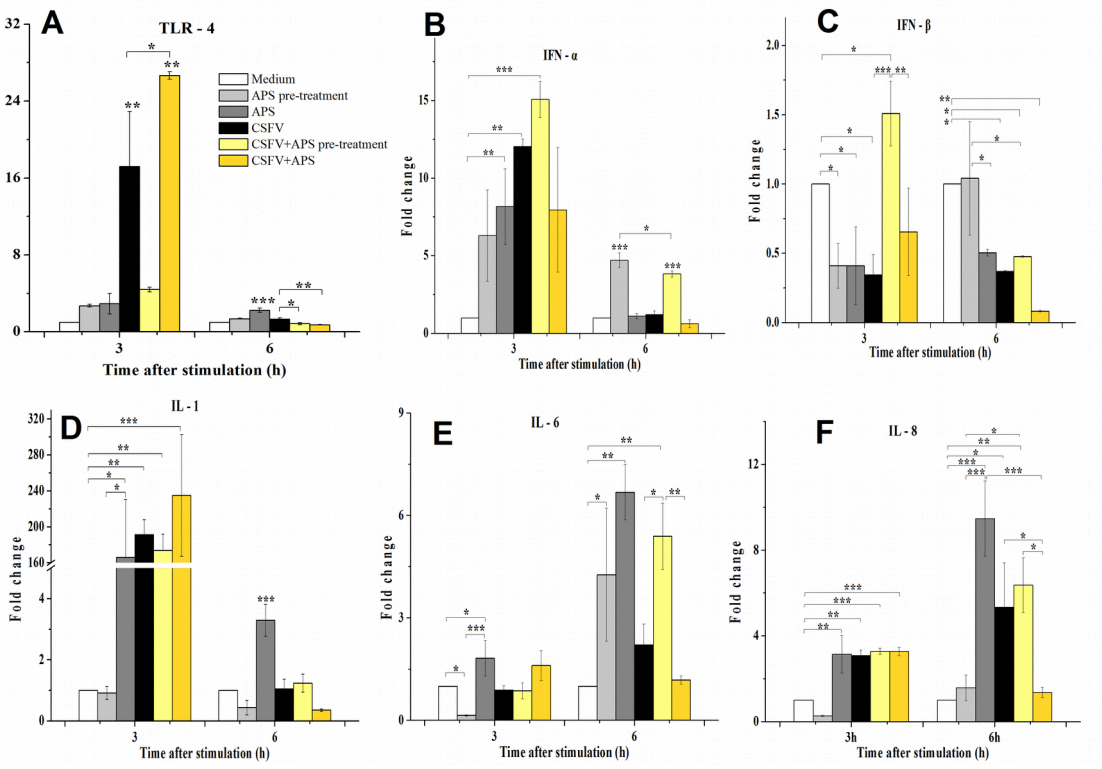
452

453 **Figure 3**



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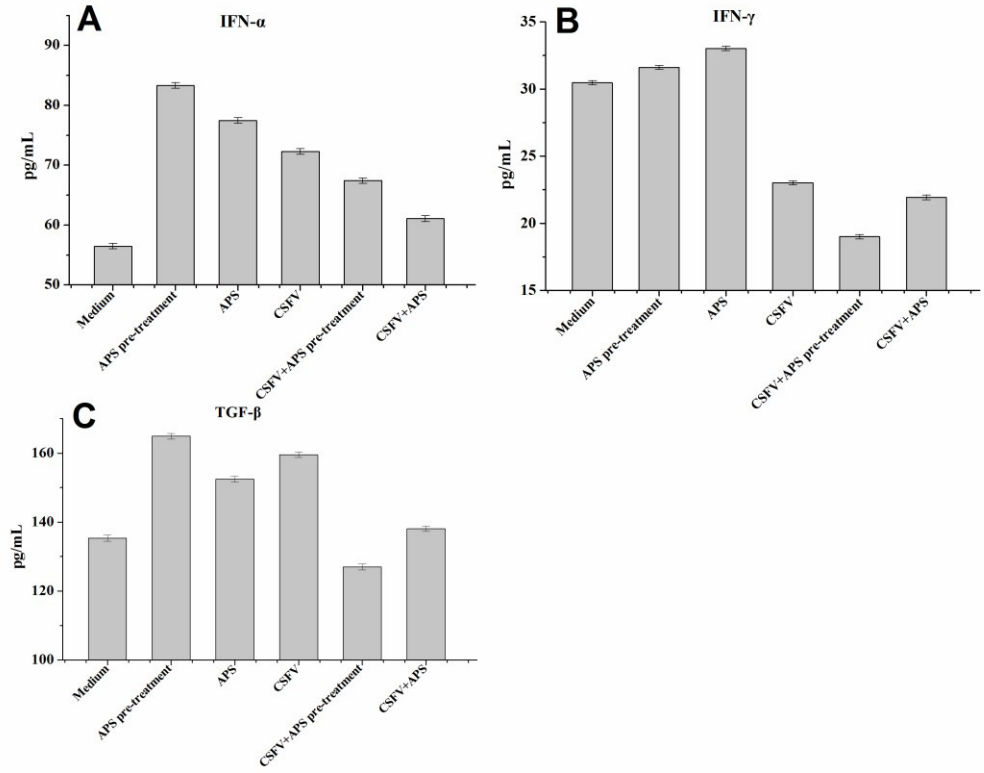
455 **Figure 4**


A
IFN-α
pg/mL
90
80
70
60
50
Medium
APS pre-treatment
APS
CSFV
CSFV+APS pre-treatment
CSFV+APS
B
IFN-γ
pg/mL
35
30
25
20
15
Medium
APS pre-treatment
APS
CSFV
CSFV+APS pre-treatment
CSFV+APS
C
TGF-β
pg/mL
160
140
120
100
Medium
APS pre-treatment
APS
CSFV
CSFV+APS pre-treatment
CSFV+APS

456