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Research Articles 研究报告

CSFV N^{pro} protein can suppress innate antiviral responses induced by poly(I:C) and influenza A virus

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Abstract: [Objective] Classical swine fever virus (CSFV) causes a highly contagious porcine disease that is characterized by hemorrhage syndrome and immunosuppression. In this paper, we studied the mechanism of CSFV inhibiting the host's innate immunity and its potential effect on influenza A virus (IAV) infection under immunosuppressive conditions. **[Methods]** First, the expression of interferons (IFNs) and IFN-stimulated genes (ISGs) and the phosphorylation of signal transducer and activator of transcription 1 (STAT1) induced by poly(I:C) in PK-15 cells which had been infected by CSFV were detected by reverse transcription-PCR (RT-PCR), reverse transcription-quantitative PCR (RT-qPCR), and Western blotting to explore the effect of CSFV on host innate immunity. Then, cells overexpressing CSFV proteins were used to screen and identify the key protein inhibiting the innate immunity. Finally, the potential influence of CSFV N^{pro} on innate immunity and IAV infection was examined in N^{pro}-overexpressing PK-15 cells by RT-PCR, RT-qPCR, Western blotting, and plaque assay. **[Results]** CSFV suppressed expression of type Ⅰ and type Ⅲ IFNs induced by poly(I:C). CSFV N^{pro} protein directly inhibited STAT1 phosphorylation and caused down-regulation of oligoadenylate synthase-like (*OASL*) protein, 2′-5′-oligoadenylate synthase-1 (*OAS1*), IFN-induced transmembrane protein 3

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(*IFITM3*), and interferon-stimulated gene 15 (*ISG15*) *in vitro*. Moreover, CSFV N^{pro} protein suppressed the expression of type Ⅰ and type Ⅲ IFNs induced by IAV, and caused a dramatic decline in phosphorylation of STAT1 and expression of *OASL*, *OAS1*, *IFITM3*, and *ISG15*, thereby significantly promoting the replication of IAV in the N^{pro}-overexpressing PK-15 cells. **[Conclusion]** CSFV N^{pro} protein antagonizes the innate antiviral response induced by poly(I:C) and IAV, suggesting that N^{pro} protein can inhibit the RIG-I-dependent signaling pathway and promote IAV replication in the N^{pro}-expressing PK-15 cells.

Keywords: classical swine fever virus (CSFV); influenza A virus (IAV); N^{pro}; innate immunity; interferon; signal transducer and activator of transcription 1

N^{pro} 蛋白可抑制 poly(I:C)和流感病毒激活的天然免疫应答

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摘 要：【目的】猪瘟病毒(classical swine fever virus，CSFV)感染猪引起出血综合征和免疫抑制，是一种高度传染性的猪病。本研究以 CSFV 为研究对象，探究 CSFV 抑制宿主天然免疫的机制及其在免疫抑制条件下对甲型流感病毒(influenza A virus，IAV)感染的潜在作用。【方法】首先，为探究 CSFV 对宿主天然免疫的影响，利用反转录 PCR (reverse transcription-PCR，RT-PCR)、荧光定量 PCR (reverse transcription-quantitative PCR，RT-qPCR)和 Western blotting 技术检测 PK-15 细胞感染 CSFV 后对 Poly(I:C)诱导干扰素(interferons，IFNs)、干扰素诱导基因(IFN-stimulated genes，ISGs)和信号传导子和转录活化子 1 (signal transducer and activator of transcription 1，STAT1)磷酸化的影响。其次，利用过表达 CSFV 蛋白的细胞系筛选并确定抑制天然免疫的关键蛋白。最后，在

过表达 CSFV N^{pro} 蛋白的细胞系上，利用 RT-PCR、RT-qPCR、Western blotting 和病毒噬斑实验技术研究 N^{pro} 蛋白对天然免疫和 IAV 感染的影响。【结果】CSFV 可抑制 poly(I:C)诱导的Ⅰ型和Ⅲ型 IFN 的表达。CSFV N^{pro} 蛋白在体外直接抑制 STAT1 的磷酸化，引起寡腺苷酸合成酶样蛋白(*OASL*)、2′,5′-寡腺苷酸合成酶 1 (*OAS1*)、干扰素诱导跨膜蛋白 3 (*IFITM3*)和干扰素刺激基因 15 (*ISG15*)表达下调。重要的是，CSFV N^{pro} 蛋白可抑制 IAV 诱导的Ⅰ型和Ⅲ型 IFN 的表达，并导致 STAT1 的磷酸化和 *OASL*、*OAS1*、*IFITM3* 和 *ISG15* 的表达显著下降，进而显著促进 IAV 在过表达 N^{pro} 蛋白的 PK-15 细胞中复制。【结论】猪瘟病毒的 N^{pro} 蛋白能够拮抗由 poly(I:C)和 IAV 所激活的天然免疫应答，表明 N^{pro} 蛋白可抑制 RIG-I 依赖的信号通路并可促进 IAV 在表达 N^{pro} 蛋白的 PK15 细胞中复制。

关键词：猪瘟病毒；甲型流感病毒；N^{pro}；天然免疫；干扰素；信号传导子和转录活化子 1 (STAT1)

Classical swine fever (CSF) is a highly contagious, World Organization for Animal Health (OIE)-listed disease caused by CSF virus (CSFV) in pigs. CSF occurs in many countries and leads to huge economic losses in the pig industry. Currently, prophylactic vaccination using lapinized live attenuated vaccine strain is the primary and effective method for the control of CSFV infections. However, it has not yet led to complete eradication of CSFV and CSF is still observed sporadically or endemically both in domestic pigs and wild boars[1–4].

CSFV belongs to the *Pestivirus* genus within the *Flaviviridae* family, and is a small enveloped, positive-sense, single-stranded RNA virus with a 12.3 kb genome surrounded by a lipid bilayer. CSFV carries a large open reading frame (ORF), encoding a single polyprotein composed of about 3 898 amino acids, flanked by 5′-UTR and 3′-UTR. The polyprotein is processed by cellular and viral proteases into 4 structural proteins (C, E^{rns}, E1, and E2) and 8 non-structural proteins (N^{pro}, P7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B). 5′-UTR and 3′-UTR are involved in the initiation of the transcription of the CSFV genome and the translation of proteins. These CSFV proteins function in the adsorption to host cells, viral replication, assembly, release of virion, and suppression of host innate antiviral responses[5–8].

Influenza A virus (IAV), which belongs to the family *Orthomyxoviridae*, consists of eight single-stranded, negative-sense gene segments. On the basis of antigenic differences of two surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA), the IAVs are divided into multiple subtypes. The H17N10 and H18N11 were detected in bats[9], while the other subtypes were all identified in avian species[10–14]. Pigs are susceptible to infection with avian, human, and swine IAVs. Among the subtypes of IAVs, H1N1, H1N2, and H3N2 are widely circulating in pigs[15–17]. More importantly, supportive evidences have shown that pigs are “mixing vessel” in the generation of novel IAV reassortants, which pose potential threat to public health. The pandemic (pdm/09) H1N1 virus emerged in 2009 clearly demonstrates the pivotal roles of pigs in new IAV outbreaks[18]. Since pigs are not vaccinated against IAVs, the virus may spread quickly and freely when novel IAVs are introduced into pig herds, especially under immunosuppressive conditions. Co-infection or multiple infection of CSFV with other swine viruses is prevalent among swine herds[19–20]. For instance, co-infection of CSFV and IAV has been reported in Henan, Shandong, Guangxi, Guangdong, and Hainan Province in China[21–27]. When invading a susceptible host, intensive immunosuppression mediated by CSFV could damage both the hematopoietic and immune systems, which makes the host easier for co-infection or secondary infection with other viruses. However, the precise mechanisms of immunosuppression induced by CSFV and their potential effect on IAVs are still unknown.

The innate immunity provides the first line of defense against pathogenic infections. On invading host, CSFV induces the expression of various cytokines, which mediate activation of innate immune signaling such as JAK-STAT pathway that is required for triggering the production of numerous antiviral factors[28]. Previous studies have shown that a series of pattern recognition receptors (PRRs) including toll-like receptor 3 (TLR3), TLR7, RIG-I, and MDA5 can sense pathogen associated molecular patterns (PAMPs) produced by CSFV to activate innate immunity signaling pathways, leading to the expression of type I IFNs[29–32]. Our previous study indicated that CSFV infection was able to induce the expression of type III IFNs including interleukin-28B (*IL-28B*) and *IL-29* in PK-15 cells and pig tissues by activating NF-κB signaling pathway[28]. Moreover, CSFV infection activated downstream of STAT1 signaling and upregulated the expression of several critical IFN-stimulated genes (ISGs) including *IFITM3*, *OASL*, *OAS1*, and *ISG15 in vitro* and *in vivo*[28]. However, the expression of type III IFNs induced by CSFV were limited to lower levels compared to the expression levels induced by IAVs[33], and therefore, we speculate that it may be associated with the immunosuppressive characteristics of CSFV.

CSFV can evade host immunosurveillance through a variety of strategies, thereby facilitating virus infection and establishing persistent infections. For example, CSFV C protein blocks the production of IFN by interacting with hemoglobin subunit beta (HB) that can antagonize CSFV replication through triggering RIG-I-mediated IFN signaling pathway[34]. E2 protein, one of the enveloped glycoproteins of CSFV, is involved in viral entry into target cells and inhibition of innate immune response in swine[35–36]. It has been demonstrated that E2 interacts with thioredoxin 2 (Trx2) to inhibit NF-κB signaling, and interacts with mitogen-activated protein kinase 2 (MAPK2) to attenuate JAK-STAT signaling[37]. Another structural protein of CSFV, E^{rns}, can antagonize extracellular dsRNA-mediated *IFN-β* expression[38].

N^{pro}, a vital non-structural protein of CSFV, has protease activity and plays an important role in escaping host immune response. N^{pro} limits the induction of *IFN-α* and *IFN-β* by promoting the proteasome degradation of IRF3[39–40]. It also counteracts IFN-α induction by interacting with IRF7 in plasmacytoid dendritic cells, and blocks poly(I:C)-induced *IFN-β* production by interacting with poly(C)-binding protein 1 (PCBP1) in HEK293T cells[41–42]. Additionally, N^{pro} directly interacts with IκBα to cause its transient accumulation in cellular nuclei[43]. Another study showed that N^{pro} could inhibit CSFV replication by modulating TLR3 expression[44]. Although progress has been made in understanding of N^{pro} protein function, mechanism underlying its inhibitory effect on innate immunity remains incompletely determined.

Pathogenesis of CSFV has been extensively studied, but the precise mechanism of how such immunosuppression can be induced by CSFV and its potential effects on subsequent IAV infection have not been fully elucidated. In the current study, we found that CSFV significantly inhibited the expression of type I and type III IFNs induced by poly(I:C) and cytokines in PK-15 cells. We observed that CSFV greatly suppressed STAT1 phosphorylation and the expression of some critical ISGs. Furthermore, we showed that N^{pro} was a key protein of CSFV involved in the inhibition of STAT1 phosphorylation and ISGs expression during CSFV infection. Although expression of type I IFNs, type III IFNs, and some critical ISGs was greatly induced by IAV, their levels were significantly reduced and thereby resulted in the enhanced replication of IAV in the presence of CSFV N^{pro} protein. These results reveal that CSFV evolves a strategy which can circumvent the innate antiviral defense and may cause potential serious infection of IAV.

1 Materials and methods

1.1 Antibodies and reagents

The primary antibodies used in this study included anti-phospho-STAT1 (Cell Signaling Technology, #7649), anti-STAT1 (Santa Cruz

Biotechnology, sc-346), anti-Flag (Proteintech, 66008-2-Ig), and anti-β-actin (TransGen Biotechnology, HC201). The rabbit anti-NP polyclonal antibody (pAb) were prepared in our laboratory as previously described[45]. The poly(I:C) was purchased from Sigma-Aldrich (P9582). The cycloheximide (CHX) was purchased from Cell Signaling Technology. Lipofectamine 2000 was obtained from Invitrogen.

1.2 Viruses and cells

The CSFV Shimen strain was a gift from Prof. Jinding Chen (The South China Agriculture University). Viral titers of CSFV were determined and calculated as described previously[28]. The IAV strain A/WSN/ 33 (H1N1) (WSN) was propagated in 10-day-old specific-pathogen-free (SPF) embryonated chicken eggs. HEK293T cells and the swine kidney cell line PK-15 were purchased from American Type Culture Collection. HEK293T cells and PK-15 cells were maintained in complete Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). All cells were incubated at 37 °C with 5% CO_2.

1.3 Poly(I:C) transfection and virus infection

PK-15 cells were transfected with poly(I:C) (0.25 or 0.5 μg/mL) for indicated time, and then the cells were used for analysis of IFNs and ISGs. PK-15 cells were transfected with poly(I:C) for 45 or 90 min, then the cells were cultured for another 5 h (namely 45 min–5 h or 90 min–5 h). The supernatant from 90 min–5 h were used for subsequent stimuli to activate innate immunity. To obtain the supernatant without cytokines, PK-15 cells were pretreated with cycloheximide (CHX) for 2 h, and then the cells were washed three times with phosphate-buffered saline (PBS) and cultured in DMEM at 37°C with 5% CO_2 for 5 h. PK-15 cells were grown to approximately 80% confluence in cell culture plates and then infected with CSFV or WSN at a multiplicity of infection (MOI) of 3 or 1, respectively. For viral infection, cells were infected with viruses for 36 h (CSFV) or 24 h (IAV) and then the cells were transfected with poly(I:C) for 5 h. Total RNA and proteins were isolated from the cells and further used for RT-PCR, RT-qPCR, and Western blotting.

1.4 Plasmid construction and overexpression study

Full-length cDNA encoding CSFV N^{pro} was subcloned into the vector pNL-CMV with a Flag tag in the N terminus to generate pNL-Flag-N^{pro}. The plasmids pNL-Flag-P7, pNL-Flag-NS2, pNL-Flag-NS3, pNL-Flag-NS4A, pNL-Flag-NS4B, pNL-Flag-NS5A, and pNL-Flag-NS5B were constructed in the same way. The specific primers with restriction enzyme sites are shown in Table 1. To construct stable cell lines overexpressing CSFV N^{pro}, HEK293T cells seeded into a 10-cm cell culture dish were co-transfected with 8 μg of pNL-Flag-N^{pro} or pNL-CMV (serving as a control), pNL-package, pNL-VSVG. At 6 h post-transfection, the cultured medium was replaced with DMEM supplemented with 10% FBS to incubate for 48 h. The packaged lentiviruses were harvested to infect PK-15 cells to construct overexpressing cell lines. The expression of Flag-labeled N^{pro} in infected PK-15 cells (PK-N^{pro} cells) was analyzed by Western blotting using anti-Flag monoclonal antibody (MAb) (dilution 1:1 000).

1.5 RT-PCR and quantitative real-time PCR

Total RNA was isolated from cultured PK-15 cells using TRIzol reagent (Invitrogen) and reverse transcribed into cDNA utilizing M-MLV Reverse Transcriptase (Promega) according to the manufacturer's instructions. The cDNA was analyzed by RT-qPCR using TransStart Green qPCR SuperMix (TransGen Biotech, AQ601-01) and RT-PCR using r*Taq* DNA polymerase (TaKaRa Bio, R001A). The amplified products by RT-PCR were resolved on 1% agarose gels, and when necessary, the intensity of bands was analyzed using Quantity One software (Bio-Rad) as previously described[46]. The primers specific for CSFV E2, N^{pro}, swine *IFN-β*, *IL-28B*, *IL-29*, *OAS1*, *OASL*, *IFITM3*, *ISG15*, and β-actin (the reference housekeeping gene including *β-actin* and *GAPDH* for internal standardization) were designed using the Primer Premier 5 software (Table 2). The data of RT-qPCR analysis were shown in normalized ratios which was auto-calculated using $\triangle\triangle C_t$ method by LightCycler system (Roche).

Table 1 Primer sequences used for PCR

Primer name	F/R	Primer sequence (5′→3′)
Flag-N^{pro}	Forward	TTTAGTGAACCGTCAGATCCGCTAGCATGGATTACAAGGACGACGATGACAAGGAGTTGAATCATTTTGAACT
	Reverse	TTGTAATCCAGAGGTTGATTCTCGAGCTAGCAACTGGTAACCCACAATG
Flag-P7	Forward	CTAGCTAGCATGGATTACAAGGACGACGATGACAAGCTACCATTGGGCCAGGGTGA
	Reverse	CCGCTCGAGCTAACCCTTGGCGACCCCGCTAA
Flag-NS2	Forward	TTTAGTGAACCGTCAGATCCGCTAGCATGGATTACAAGGACGACGATGACAAGGGAAAGATAGATGGCGGTTG
	Reverse	TTGTAATCCAGAGGTTGATTCTCGAGCTATCTAAGCACCCAGCCAAGGT
Flag-NS3	Forward	CTAGCTAGCATGGATTACAAGGACGACGATGACAAGGGGCCTGCCGTTTGCAAGAA
	Reverse	CCGCTCGAGCTATAGACCAACTACTTGTTTTA
Flag-NS4A	Forward	CTAGCTAGCATGGATTACAAGGACGACGATGACAAGTCAACAGCTGAGAATGCCTT
	Reverse	CCGCTCGAGCTATAGCTCCTTCAATTCTGTCT
Flag-NS4B	Forward	CTAGCTAGCATGGATTACAAGGACGACGATGACAAGGCTCAGGGGGATGTGCAGAG
	Reverse	CCGCTCGAGCTATAGCTGGCGGATCTTTCCTT
Flag-NS5A	Forward	CTAGCTAGCATGGATTACAAGGACGACGATGACAAGTCAAGTAATTACATTCT
	Reverse	CCGCTCGAGCTACAGTTTCATAGAATACA
Flag-NS5B	Forward	CTAGCTAGCATGGATTACAAGGACGACGATGACAAGAGTAATTGGGTGATGCA
	Reverse	CCGCTCGAGTCATACCCCTCTCCCTATCA

Table 2 Primer sequences used for RT-PCR and RT-qPCR

Primer name	F/R	Primer sequence (5′→3′)
β-actin	Forward	GACCTGACCGACTACCTCAT
	Reverse	CGTAGAGGTCCTTCCTGATGT
GAPDH	Forward	TTCACGACCATGGAGAAGGC
	Reverse	GGACACAACCTGGTCCTCAG
IFN-β	Forward	ACAAAGGAGCAGCAATTTGGC
	Reverse	TCTGCCCATCAAGTTCCACAA
IL-28B	Forward	CTCCTTGGCGAACTCATCCC
	Reverse	TCTCCACTGGCGACACATTT
IL-29	Forward	CATGGGCCAGTTCCAATCTCT
	Reverse	CTGATGCAAGCCTGAAGTTCG
OASL	Forward	CCCACAAGGAGTGTAAAGAAGA
	Reverse	GGCCTCAATCAGATCCACATAG
OAS1	Forward	GGAAGCCATCGACATCGTCT
	Reverse	GGGCAGGACATCAAACTCCA
IFITM3	Forward	CATATGAGATGCTCAAGGAGGAG
	Reverse	CAGTGGTGCAAACGATGATG
ISG15	Forward	GTTGATGGTGCAAAGCTTCAG
	Reverse	CACATAGGCTTGAGGTCATACTC
CSFV-E2	Forward	CGGCAACACAACTGTCAAGG
	Reverse	AGCGGCGAGTTGTTCTGTTA
CSFV-N^{pro}	Forward	TTGGAGACCCGAGTGAGGTA
	Reverse	CGCAAAACTGCGCTTCGTTA
NP	Forward	TCAAACGTGGGATCAATG
	Reverse	GTGCAGACCGTGCTAAAA

1.6 Western blotting

Western blotting was performed as described previously[47]. Briefly, PK-15 cells were incubated on ice with RIPA lysis buffer (Cell Signaling Technology, #9803) containing 1 mmol/L PMSF (Beyotime, ST506) for 30 min. The lysates were clarified by centrifugation at 12 000 r/min for 10 min at 4 °C, and the protein concentration was quantified by the BCA protein assay kit (Beyotime, P0012). Equal amounts of protein samples were separated on 12% SDS-PAGE gels, transferred onto NC membranes (Merck Millipore, HATF00010) and blocked with 5% (*W/V*) milk powder in Tris-buffered saline (TBS, pH 7.4) for 2 h at room temperature. The membranes were then probed with indicated primary antibodies for 2.5 h at room temperature. The primary antibodies used in Western blotting included anti-phospho-STAT-1 (dilution 1:2 000), anti-STAT-1 (dilution 1:5 000), anti-Flag (dilution 1:1 000), and anti-β-actin (dilution 1:5 000). Next, the membranes were washed 3 times in TBS, followed by incubation with appropriate secondary antibodies conjugated to horseradish peroxidase (HRP) at room temperature for 2 h. The signals were detected by the Protein Simple Fluor Chem M system (Protein Simple) after incubation with ECL Plus (Thermo Fisher Scientific, 34095).

1.7 Enzyme-linked immunosorbent assay (ELISA)

Cell culture supernatants were collected from the culture medium of PK-15 cells transfected with poly(I:C) at indicated time points as a source of poly(I:C)-induced cytokines. To quantify production of *IFN-β* and *IL-29*, cell culture supernatants was harvested and examined by using the pig *IFN-β* or *IL-29* ELISA Kit (Shanghai Jianglai Biological Technology) according to manufacturer's instruction.

1.8 Plaque assay

MDCK cells were seeded in 6-well plates overnight and then incubated with serial dilutions of viral supernatants for 2 h. After incubation, the cells were washed and overlaid with DMEM containing 0.6% low-melting-point agarose and 2 μg/mL TPCK-trypsin at 4 °C for 30 min. Following this, the plates were placed upside down at 37 °C for a further 72 h. Visible plaques were counted for viral titer determination.

1.9 Statistical analysis

Results were shown as mean values±standard error (mean±SE). Statistical significance was determined by Student's *t*-test analysis. A level of $P<0.05$ was considered to be significant.

2 Results and analysis

2.1 CSFV inhibits the expression of type I and type III IFNs induced by poly(I:C) in PK-15 cells

Previous studies showed that CSFV was able to induce expression of type I and type III IFNs, but the expression of type III IFNs were limited to lower levels[28,48]. Therefore, we speculate that CSFV evolves a mechanism that can inhibit the expression of IFNs. The results showed that poly(I:C) was able to induce the expression of IFN-β, *IL-28B*, and *IL-29* in PK-15 cells (Figure 1A). Further analysis indicated that the poly(I:C)-induced expression of *IFN-β*, *IL-28B*, and *IL-29* was significantly impaired by infection of CSFV in PK-15 cells, as compared with that in CSFV non-infected PK-15 cells (Figure 1B). These findings were further confirmed by independent analysis using quantitative real-time PCR (RT-qPCR) (Figure 1C–E). These results suggest that CSFV may exert a strong inhibitory effect on the expression of type I and type III IFNs induced by innate immune signaling upon stimulation of poly(I:C) in swine cells.

2.2 CSFV impairs phosphorylation of STAT1 and expression of several critical ISGs induced by poly(I:C) in PK-15 cells

JAK/STAT pathway, activated by cytokines such as type I and type III IFNs, plays crucial

roles in innate immunity, thereby protecting the host from pathogen infections[49–50]. Since poly(I:C)-induced expression of type I and type III IFNs was suppressed by CSFV in PK-15 cells, we assumed that the downstream signaling should be inhibited during CSFV infection. To address this, we investigated the phosphorylation level of STAT1 during CSFV infections in PK-15 cells. As expected, the phosphorylation level of STAT1 induced by poly(I:C) was markedly reduced in CSFV infected PK-15 cells, as compared with that in control cells (Figure 2A–B). Moreover, the expression of some key ISGs including *OASL*, *OAS1*, *IFITM3*, and *ISG15* was significantly induced after transfection with poly(I:C) in PK-15 cells (Figure 2C), but such induction was clearly inhibited by CSFV, as evidenced by decreased levels of these ISGs in the CSFV infected cells compared with that in the control cells (Figure 2D). In addition, the results were further confirmed by independent analysis of RT-qPCR assays (Figure 2E–H). Taken together, these data reveal that CSFV had a strong inhibitory effect on the phosphorylation of STAT1 and the expressions of *OASL*, *OAS1*, *IFITM3*, and *ISG15* induced by poly(I:C) in PK-15 cells.

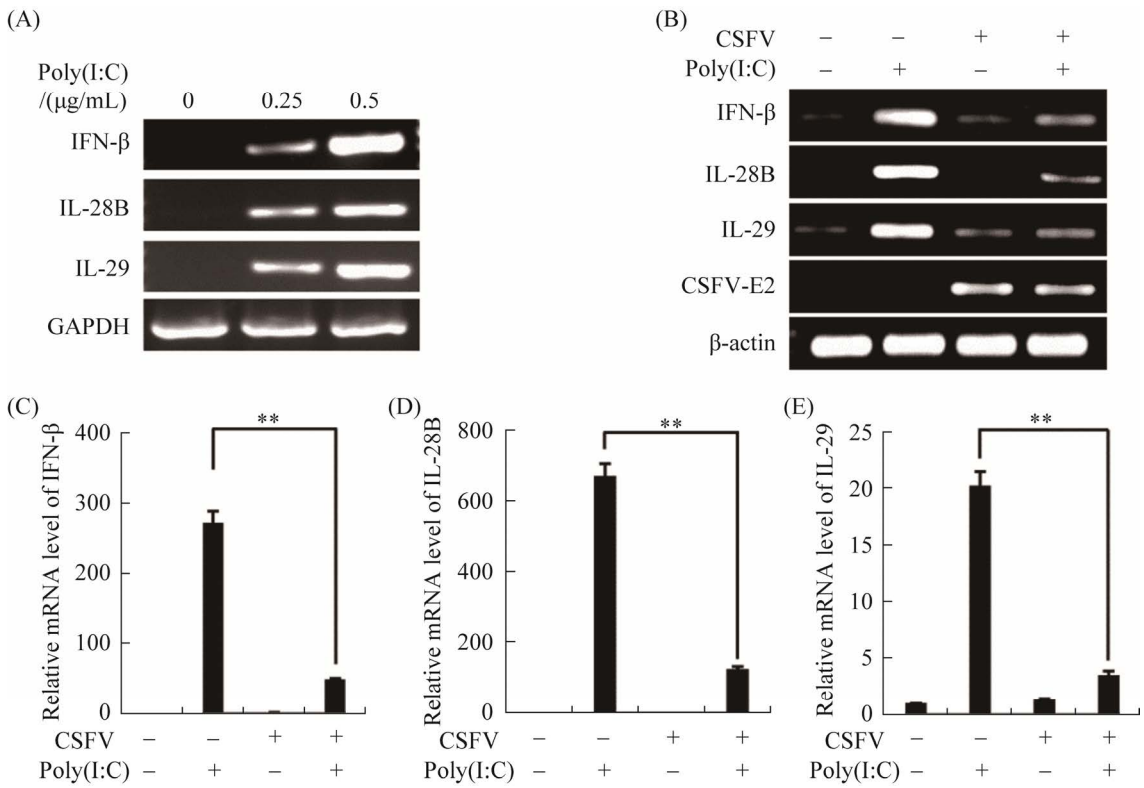

Figure 1 CSFV significantly inhibits the expression of type I and III IFNs induced by poly(I:C) in PK-15 cells. A: PK-15 cells were transfected with poly(I:C) using Lipofectamine 2000 (L2000) at a final concentration of 0.25 or 0.50 μg/mL for 5 h; the expressions of *IFN-β*, *IL-28B*, and *IL-29* in the cells were determined by RT-PCR assay; B: PK-15 cells infected with or without CSFV for 36 h, and then the cells were transfected with poly(I:C) (0.50 μg/mL) for 5 h. The expressions of *IFN-β*, *IL-28B* and *IL-29* were examined by RT-PCR assay. PK-15 cells infected with or without CSFV were treated as described in (B). Then RT-qPCR was performed to examine the mRNA levels of *IFN-β* (C), *IL-28B* (D), and *IL-29* (E), respectively. Plotted are the average results from three independent experiments. **: $P<0.01$.

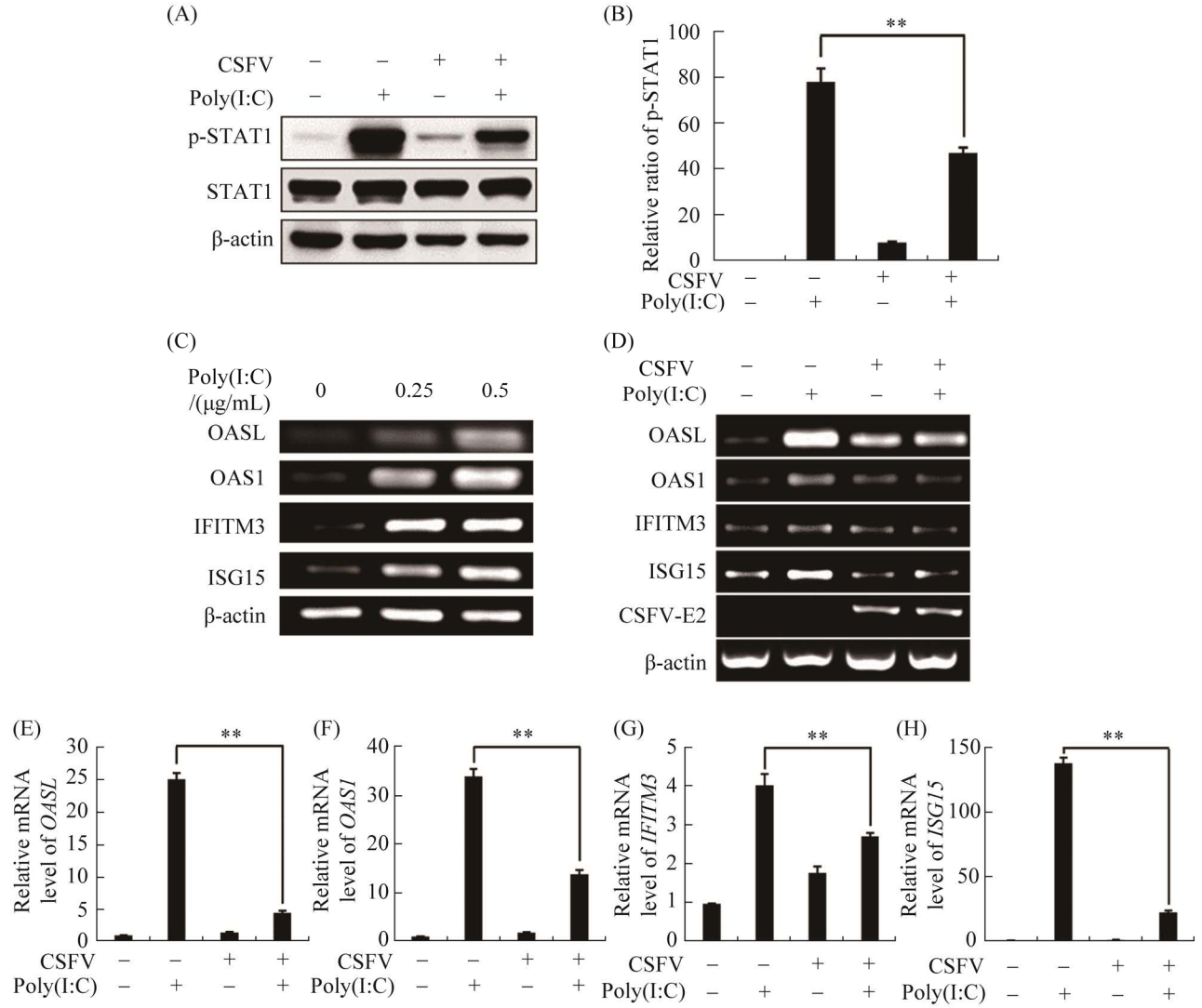

Figure 2 CSFV inhibits phosphorylation of STAT1 and expression of ISGs induced by poly(I:C) in PK-15 cells. A: PK-15 cells infected with or without CSFV for 36 h, and then the cells were transfected with poly(I:C) (0.50 μg/mL) for 5 h. STAT1 and phosphorylation of STAT1 were analyzed by Western blotting using the indicated antibodies; B: the band ratio of phosphorylation of STAT1 in (A) was analyzed using image J software. The endogenous β-actin was used as an internal control; C: PK-15 cells were transfected with poly(I:C) using Lipofectamine 2000 (L2000) at a final concentration of 0.25 or 0.50 μg/mL for 5 h; the expression of *OASL*, *OAS1*, *IFITM3*, and *ISG15* in the cells was determined by RT-PCR assay; D: PK-15 cells infected with or without CSFV were treated as described in (A). The expressions of *OASL*, *OAS1*, *IFITM3*, and *ISG15* were examined by RT-PCR assay. PK-15 cells infected with or without CSFV were treated as described in (A). Then RT-qPCR was performed to examine the mRNA levels of *OASL* (E), *OAS1* (F), *IFITM3* (G), and *ISG15* (H), respectively. Plotted are the average results from three independent experiments. **: $P<0.01$.

2.3 CSFV ihibits STAT1 phosphorylation and ISGs expression induced by cytokines in PK-15 cells

Next, we examined the effects of CSFV infection on cytokines-activated STAT1 phosphorylation and ISGs expression. First, time course expression of type I and type III IFNs in PK-15 cells stimulated by poly(I:C) was analyzed

by using RT-PCR and ELISA. We observed that the expression of IFN-β, *IL-28B*, and *IL-29* was slightly increased at 45 min, while the expression of the cytokines was significantly upregulated at 90 min, 4 h, and 6 h after transfection with poly(I:C) (Figure 3A). Furthermore, PK-15 cells were transfected with poly(I:C) for 45 or 90 min and then the cell culture medium was replaced with DMEM without poly(I:C). The PK-15 cells were then cultured for another 5 h (namely 45 min–5 h or 90 min–5 h). The results showed that expression of IFN-β, *IL-28B*, and *IL-29* were significantly induced in 90 min–5 h cells as compared with that in 45 min–5 h cells (Figure 3 B–D). Next, cell culture supernatants were collected from the 45 min–5 h cells or 90 min–5 h cells and PK-15 cells were treated with these supernatants for indicated times. We observed that expression of *OASL*, *OAS1*, *IFITM3*, and *ISG15* was upregulated in PK-15 cells treated with supernatant collected from 90 min–5 h cells (Figure 3E**)**. These results suggest that the supernatant collected from 90 min–5 h cells may contain cytokines such as type I and type III IFNs and thus was chosen for the further study.

To obtain the supernatant without cytokines, the PK-15 cells were pretreated with cycloheximide (CHX), a translation inhibitor to block the *de novo* synthesis of proteins, and the cells were then transfected with poly(I:C) for 90 min. Next cell culture medium was replaced with DMEM and cells were incubated for another 5 h. Subsequently, the supernatant was collected to treat naive PK-15 cells for 45 min. Western blotting and RT-PCR analysis indicated that the phosphorylation of STAT1 and expression of some key ISGs including *OASL*, *OAS1*, and *ISG15* were not stimulated due to lack of cytokines by the treatment with CHX; in contrast, the supernatant collected from the poly(I:C)-transfected PK-15 cells but without CHX-pretreatment could significantly induce the phosphorylation of STAT1 and the expression of ISGs (Figure 4A). These data indicated that the culture supernatant from PK-15 cells that contained various cytokines was able to activate the JAK-STAT signaling pathway and transcriptionally induce ISGs expressions.

To determine whether the activation of JAK-STAT signaling induced by cytokines was suppressed in the presence of CSFV, PK-15 cells were infected with CSFV and the cells were then treated with the supernatant collected from 90 min–5 h poly(I:C)-transfected cells for 45 min. The results demonstrated that the phosphorylation of STAT1 (Figure 4B) and expression of some key ISGs including *OASL*, *OAS1*, *IFITM3*, and *ISG15* (Figure 4C–F, Figure 3F**)** were significantly reduced in the CSFV infected cells, as compared with that in non-infected cells. These data suggest that CSFV was able to directly suppress the activation of the JAK-STAT signaling pathway activated by cytokines in PK-15 cells.

2.4 CSFV Npro protein suppresses the expression of type I and type III IFNs

Previous reports revealed that non-structural proteins of CSFV play important roles in counteracting innate immunity during viral infection[6,33,47,51]. To investigate which kind of non-structural proteins of CSFV could inhibit the expression of IFNs, we generated PK-15 cell lines that stably overexpressed N^{pro}, P7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B, respectively. The PK15 cells overexpressing the non-structural proteins were transfected with poly(I:C) and cultured for 5 h. Then, the IFN-β and *IL-28B* were examined by RT-PCR assay. We observed that the expression of *IFN-β* and *IL-28B* was significantly suppressed in cells overexpressing the N^{pro} protein compared with that in other CSFV non-structural protein-overexpressing cells (Figure 5A).

CSFV N^{pro} protein does not directly alter the replication kinetics in cells[52], but it has protease activity and plays a key role in the life cycle of CSFV through antagonizing host innate immunity. To confirm the inhibitory effects of N^{pro} on type I and type III IFNs expression, the PK-15 cells stably overexpressing N^{pro} protein (designated as pNL-N^{pro}

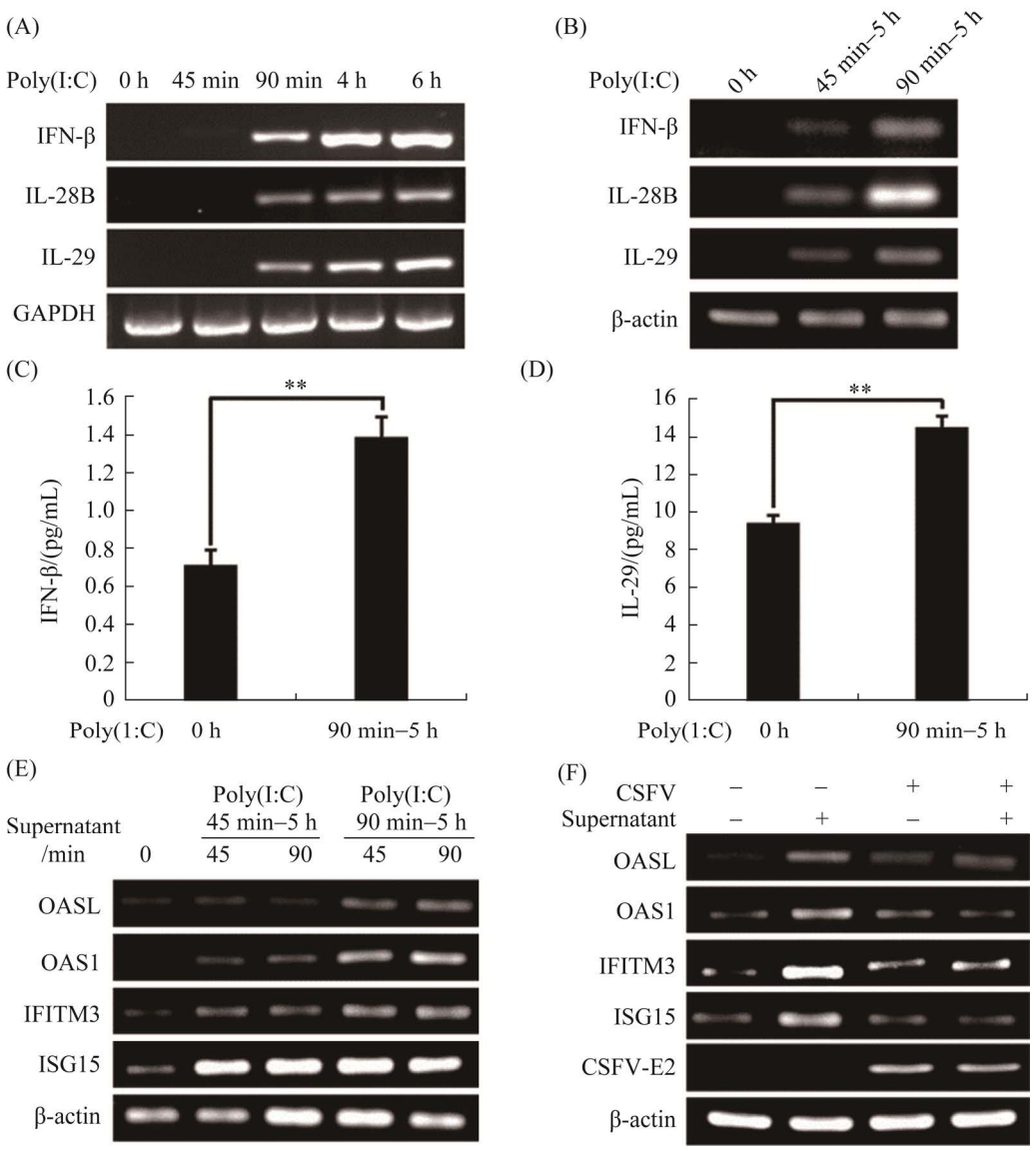

Figure 3 IFNs and ISGs expression induced by poly(I:C) or supernatant at different time points. A: PK-15 cells were transfected with poly(I:C) using Lipofectamine 2000 (L2000) at a final concentration of 0.5 μg/mL for 0 h, 45 min, 90 min, 4 h, and 6 h, respectively. Then the mRNA expression of *IFN-β*, *IL-28B*, and *IL-29* in the cells was determined by RT-PCR assay. B: PK-15 cells were transfected with poly(I:C) for 45 min or 90 min, and then the culture supernatant was replaced with DMEM for 5 h. Expression of the indicated genes was examined by RT-PCR assay. PK-15 cells were transfected with poly(I:C) for 90 min, and then the culture supernatant was replaced with DMEM for 5 h. The production of *IFN-β* (C) and *IL-29* (D) collected from the supernatant was quantified by ELISA. E: PK-15 cells were transfected with poly(I:C) using Lipofectamine 2000 (L2000) at a final concentration of 0.5 μg/mL for 45 min or 90 min, and then the culture supernatant was replaced with DMEM for 5 h; subsequently, the supernatant was collected to treat naive PK-15 cells for 45 min or 90 min. Expression of the indicated genes was examined by RT-PCR assay. CSFV inhibited the expression of ISGs induced by cytokines. F: PK-15 cells, infected with or without CSFV for 36 h, were stimulated using the culture supernatant from poly(I:C)-transfected PK-15 cells. After 45 min treatment, the expression of the indicated genes was examined by RT-PCR assay. **: $P<0.01$.

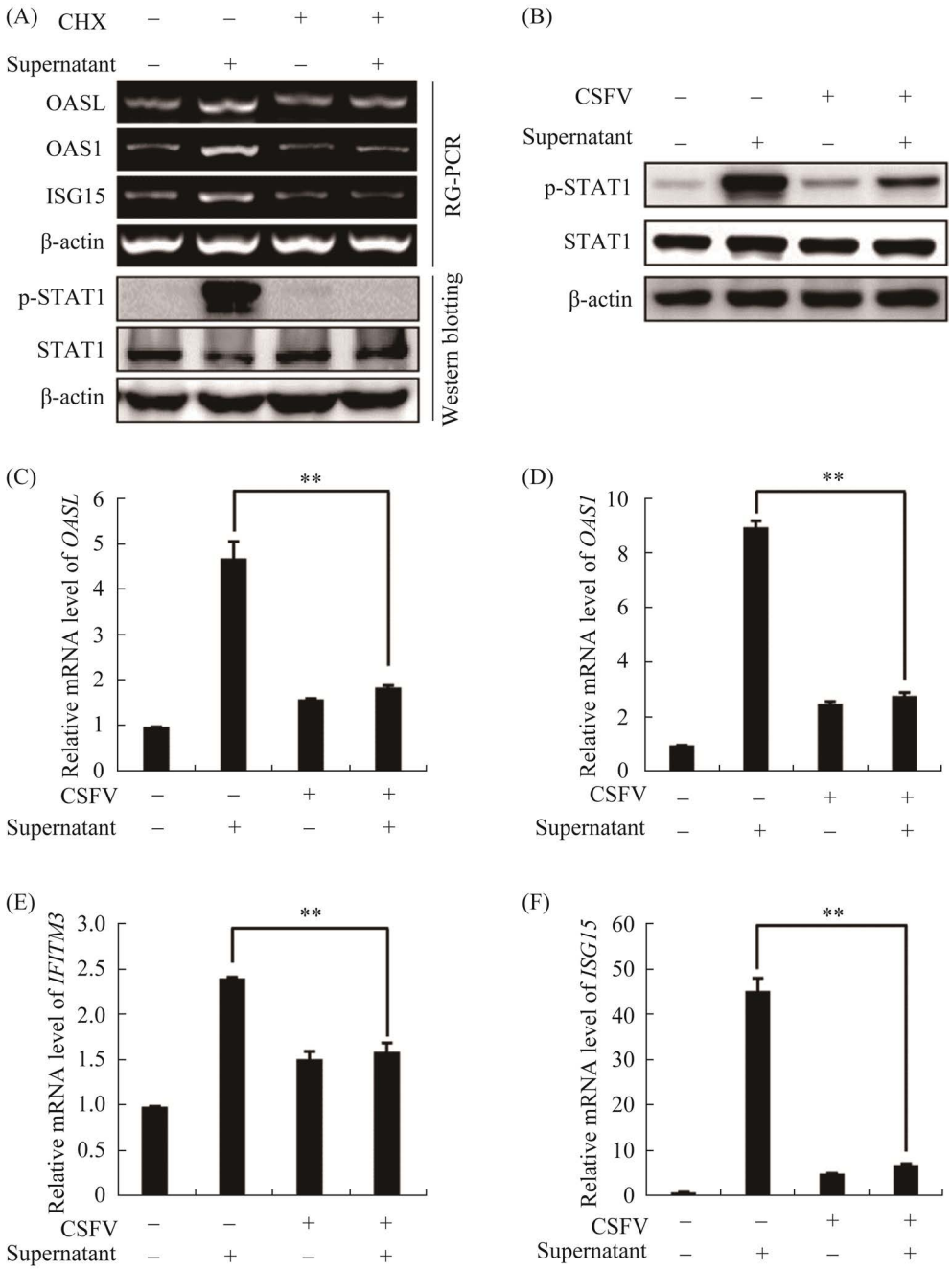

Figure 4 CSFV impairs STAT1 phosphorylation and ISGs expression induced by cytokines in PK-15 cells. A: after PK-15 cells were pretreated with protein synthesis inhibitor (CHX, 100 μg/mL) or DMSO (control) for 2 h, they were transfected with poly(I:C) for 90 min, and then the supernatant of cultured cells was replaced with DMEM for 5 h. Subsequently, the supernatant was taken to treat naive PK-15 cells for 45 min. The expression of the indicated genes was examined by RT-PCR assay and cell lysates were analyzed by Western blotting using the indicated antibodies. B: PK-15 cells, infected with or without CSFV for 36 h, were stimulated by the culture supernatant from the poly(I:C)-transfected PK-15 cells. After treatment for 45 min, the cell lysates were analyzed by Western blotting using the indicated antibodies. PK-15 cells, infected with or without CSFV, were treated as described in (B). Then the expression of *OASL* (C), *OAS1* (D), *IFITM3* (E), and *ISG15* (F) was examined by RT-qPCR. Plotted are the average results from three independent experiments. **: $P<0.01$.

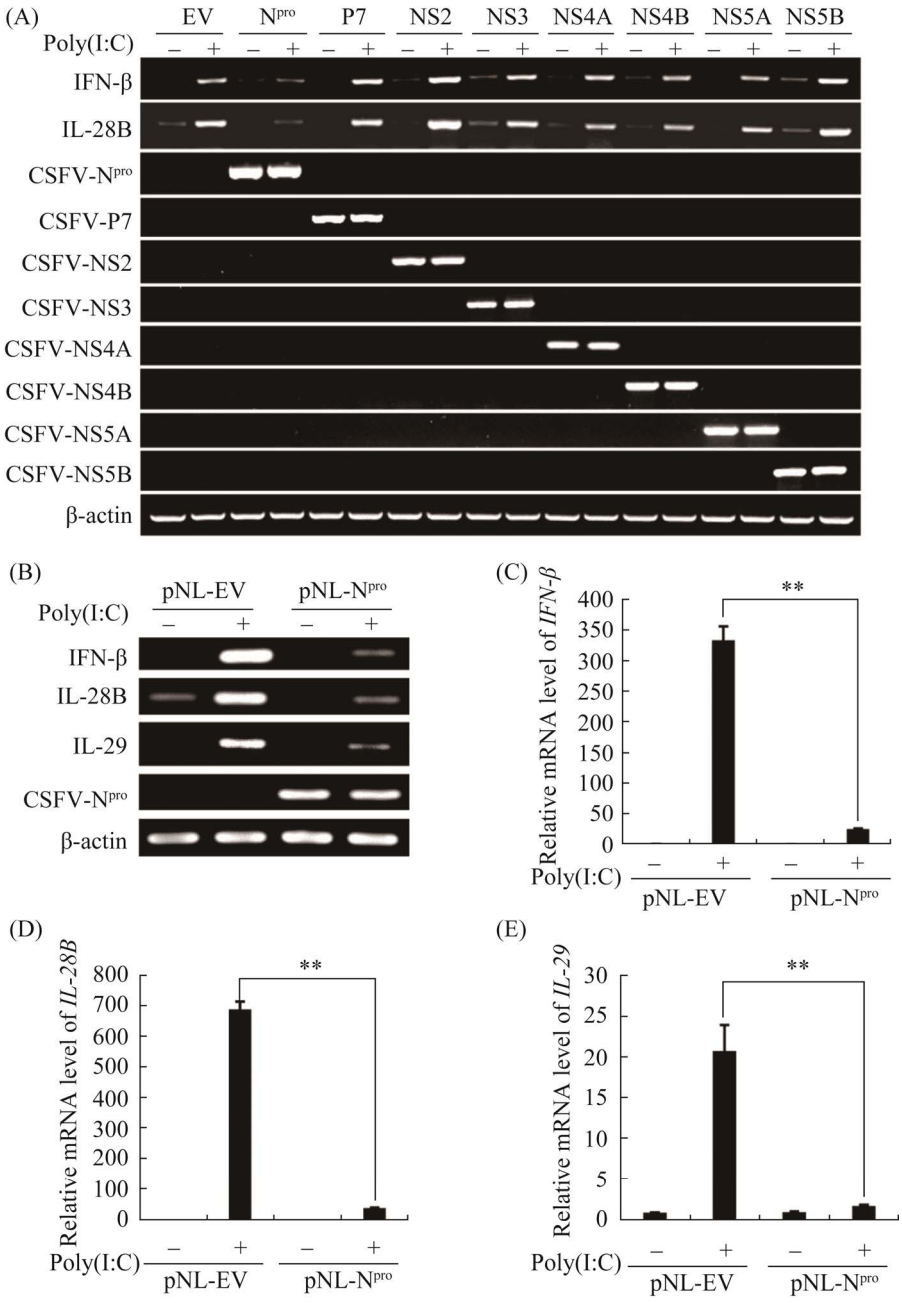

Figure 5 CSFV N^{pro} protein suppresses the expression of Type I and type III IFNs. A: the inhibition of IFNs expression by CSFV non-structural proteins. PK-15 cells stably overexpressing CSFV non-structural proteins Npro, P7, NS2, NS3, NS4A, NS4B, NS5A, NS5B, or empty vector were transfected with poly(I:C) (0.5 μg/mL) for 5 h. Expressions of *IFN-β*, *IL-28B*, and CSFV non-structural protein genes were examined by RT-PCR assay. B: PK-15 cells stably overexpressing CSFV N^{pro} protein (designated as pNL-N^{pro} cells) or empty vector (designated as pNL-EV cells) were transfected with poly(I:C) (0.5 μg/mL) for 5 h. Then the expression of *IFN-β*, *IL-28B*, and *IL-29* was examined by RT-PCR assay, respectively. pNL-N^{pro} cells or pNL-EV cells were treated as described in (B). Then the mRNA levels of *IFN-β* (C), *IL-28B* (D), and *IL-29* (E) were examined by RT-qPCR. Plotted are the average results from three independent experiments. **: $P<0.01$.

cells) were transfected with poly(I:C), cultured for 5 h, and the expression of *IFN-β*, *IL-28B*, and *IL-29* was examined by RT-PCR and RT-qPCR assays. The results from RT-PCR displayed that the expression of *IFN-β*, *IL-28B*, and *IL-29* induced by poly(I:C) were significantly decreased in the presence of N^{pro} protein, as compared with that in the control cells (designated as pNL-EV cells) (Figure 5B). The downregulation of *IFN-β*, *IL-28B*, and *IL-29* was further confirmed by using RT-qPCR analysis (Figure 5C–E). Taken together, these results suggest that N^{pro} protein of CSFV suppressed the expression of type I and type III IFNs induced by poly(I:C)-activated innate immune signaling.

2.5 CSFV Npro protein inhibits the phosphorylation of STAT1 and the expression of ISGs induced by poly(I:C)

To investigate the effect of N^{pro} on IFN downstream signaling, the pNL-N^{pro} cells were transfected with poly(I:C) and cultured for 5 h. Then the cells were harvested to assess phosphorylation of STAT1 by Western blotting, and the expression of some key ISGs by RT-PCR and RT-qPCR. The results indicated that the phosphorylation of STAT1 (Figure 6A) and the expression of *OASL*, *OAS1*, *IFITM3*, and *ISG15* examined by RT-PCR (Figure 6B) were significantly impaired in the pNL-N^{pro} cells, as compared with that in the pNL-EV cells. RT-PCR

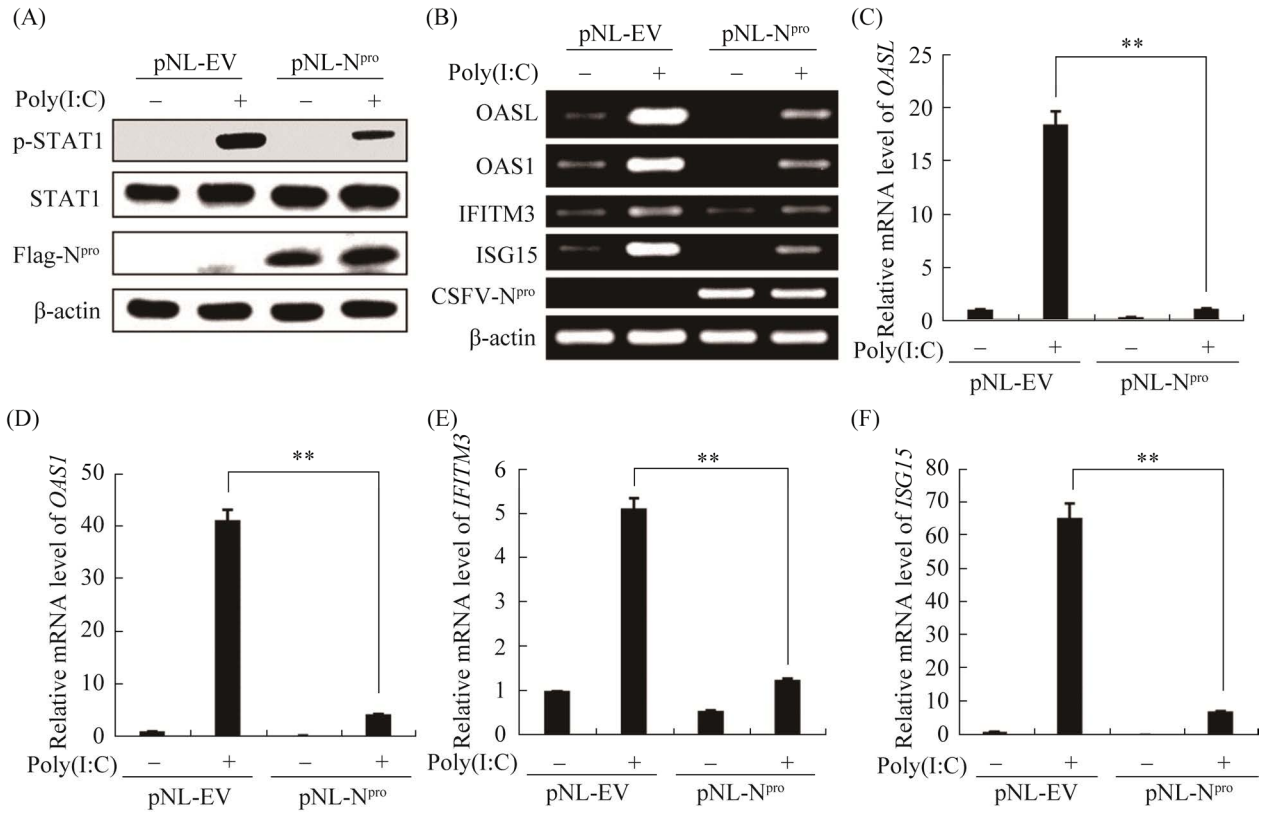

Figure 6 CSFV N^{pro} protein inhibits the phosphorylation of STAT1 and the expression of ISGs induced by poly(I:C) in PK-15 cells. A: pNL-N^{pro} or pNL-EV cells were transfected with poly(I:C) (0.5 μg/mL) for 5 h. Then the cell lysates were analyzed by Western blotting using the indicated antibodies. B: pNL-N^{pro} or pNL-EV cells were treated as described in (A). Then the expression of the indicated genes was examined by assay. pNL-N^{pro} cells or pNL-EV cells were treated as described in (B). Then the mRNA expression of *OASL* (C), *OAS1* (D), IFITIM3 (E), and *ISG15* (F) was examined by RT-qPCR, respectively. Plotted are the average results from three independent experiments. **: $P<0.01$.

similarly, analysis by RT-qPCR showed that the expression of the four ISGs were significantly downregulated in the presence of N^{pro} protein (Figure 6C–F). Collectively, these data indicate that CSFV N^{pro} can cause a dramatic decline in phosphorylation of STAT1 and expression of *OASL*, *OAS1*, *IFITM3*, and *ISG15* induced by poly(I:C).

2.6 CSFV N^{pro} protein negatively regulates the phosphorylation of STAT1 and the expression of ISGs induced by cytokines

Since CSFV suppressed the cytokine-activated JAK-STAT signaling pathway in PK-15 cells, we speculated that the inhibitory effect might be caused by viral N^{pro} protein. To test this possibility, the pNL-N^{pro} cells were treated for 45 min with the supernatant collected from 90 min–5 h poly(I:C)-transfected cells which contained cytokines, then the phosphorylation of STAT1 and ISGs were examined by Western blotting and RT-PCR, respectively. The results exhibited that the phosphorylation of STAT1 and the expression of *OASL*, *OAS1*, *IFITM3*, and *ISG15* were significantly reduced in the pNL-N^{pro} cells, as compared with that in the pNL-EV cells (Figure 7A–B). Similarly, the results from RT-qPCR showed that the expression of OASL, *OAS1*, *IFITM3*, and *ISG15* were significantly downregulated in the presence of N^{pro} protein (Figure 7C–F). These results revealed that N^{pro} protein was involved in the inhibition of the JAK-STAT signaling pathway activated by cytokines during CSFV infection, leading to a significantly declined expression of some key ISGs.

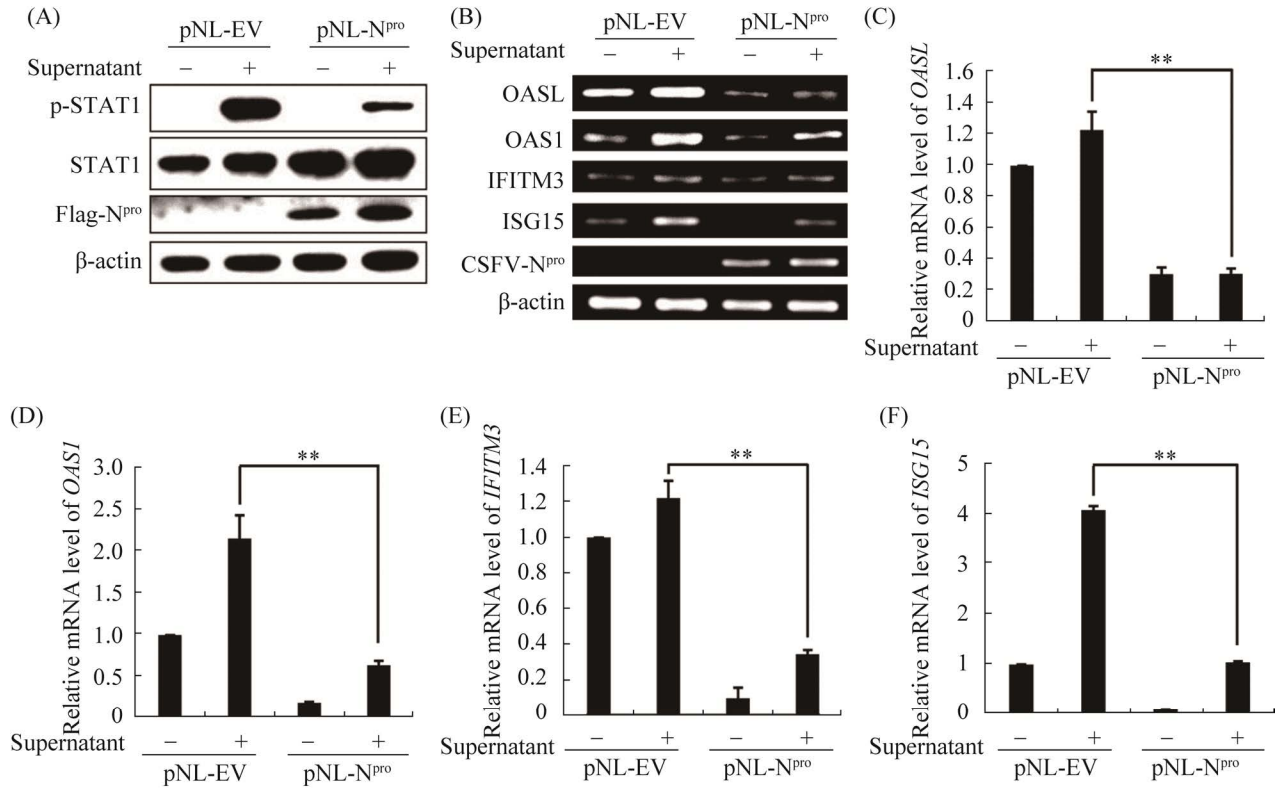

Figure 7 CSFV N^{pro} protein negatively regulates STAT1 phosphorylation and ISGs expression induced by cytokines and IFNs. A: pNL-N^{pro} or pNL-EV cells were stimulated by culture supernatant from poly(I:C)-transfected PK-15 cells for 45 min. Then the cell lysates were analyzed by Western blotting using the indicated antibodies. B: pNL-N^{pro} or pNL-EV cells were treated as described in (A). Then the expression of the indicated genes was examined by RT-PCR. pNL-N^{pro} or pNL-EV cells were treated as described in (B). Then the mRNA expression of *OASL* (C), *OAS1* (D), *IFITM3* (E), and *ISG15* (F) was examined by RT-qPCR, respectively. Plotted are the average results from three independent experiments. **: $P<0.01$.

2.7 CSFV Npro protein enhances IAV replication by suppressing type I and type III IFNs

Since N^{pro} protein of CSFV impaired the innate immunity, we determined whether the host could become more susceptible to IAV infection under immunosuppressive condition caused by CSFV. To this end, effect of CSFV N^{pro} on IAV infection was examined in the pNL-N^{pro} cells. The cells were infected with WSN virus and harvested 24 h post infection (p.i.). The results displayed that N^{pro} significantly promoted the expression of viral NP mRNA and NP protein by RT-PCR (Figure 8A), RT-qPCR (Figure 8B), and Western blotting (Figure 8C). To further determine the effect of N^{pro} on the production of influenza progeny viruses, we infected the pNL-N^{pro} cells with WSN virus and cell supernatant was harvested 24 h p.i. for titer determination with plaque assays. Consistent with the above data, the experiments demonstrated that N^{pro} strongly increased the titers of IAV compared with that in the control cells, indicating that CSFV N^{pro} can promote the IAV replication (Figure 8D). Since N^{pro} suppressed the expression of type I and type III IFNs, we speculate that the increased IAV replication might be due to reduced levels of IFNs. Thus, we detected the expressions of *IFN-β*, *IL-28B*, and *IL-29* in pNL-N^{pro} cells infected with WSN virus. As expected, results obtained from the RT-PCR and RT-qPCR showed that the expression of type I and type III IFNs were significantly inhibited compared with that in the control treatment (Figure 8E–H). Collectively, these results indicate that CSFV N^{pro} protein may enhance IAV replication through downregulation of type I and type III IFNs.

2.8 CSFV Npro protein inhibits IAV-induced STAT1 phosphorylation and the expression of ISGs

To investigate the effect of N^{pro} on IFN downstream signaling, the pNL-N^{pro} cells were infected with WSN virus, and the cells were harvested to assess STAT1 phosphorylation and some key ISG expression. We observed that the phosphorylation of STAT1 (Figure 9A) and the expression of *OASL*, *OAS1*, *IFITM3*, and *ISG15* were significantly reduced in the pNL-N^{pro} cells after infection with the IAV (Figure 9B). Similarly, analysis by RT-qPCR showed that the IAV-induced expression of these ISGs were significantly downregulated in the presence of N^{pro} protein (Figure 9C–F). Together, these data indicate that CSFV N^{pro} can cause a dramatic decline in phosphorylation of STAT1 and expression of *OASL*, *OAS1*, *IFITM3*, and *ISG15* induced by IAV.

3 Discussion

CSFV is a typical immunosuppressive virus and may evolve multiple mechanisms to inhibit the expression of cytokines to escape antiviral immunity in pigs, leading them susceptible to infection with multiple pathogens. Coinfections of multiple viruses are frequently found in pig farms. The first infection with a pathogen usually changes the host immune response against the second pathogen, which might cause a more virulent secondary infection, leading to more serious consequences[53]. Many studies have shown that CSFV interferes with the production of type I IFNs by reducing proteasomal degradation of IRF3[39,54–55]. More importantly, our previous study demonstrated that the infection with CSFV was able to induce limited expression of type III IFNs, indicating that CSFV might have evolved a strategy to inhibit the expression of type III IFNs[28]. Cao et al. found that CSFV N^{pro} protein suppressed type III IFN by reducing the expression of IRF1 and blockading its nuclear translocation[56]. However, the possible mechanisms of immunosuppression induced by CSFV and their potential effect on IAVs are still unknown. Here, the present study demonstrates that the CSFV N^{pro} protein suppressed the expression of type I and type III IFNs, resulting in the reduction of phosphorylation STAT1 and several critical ISGs and finally enhanced IAV replication in swine cells. Thus, our study has revealed that CSFV evolves a strategy to circumvent the innate antiviral defense and may cause potential serious infection of IAV.

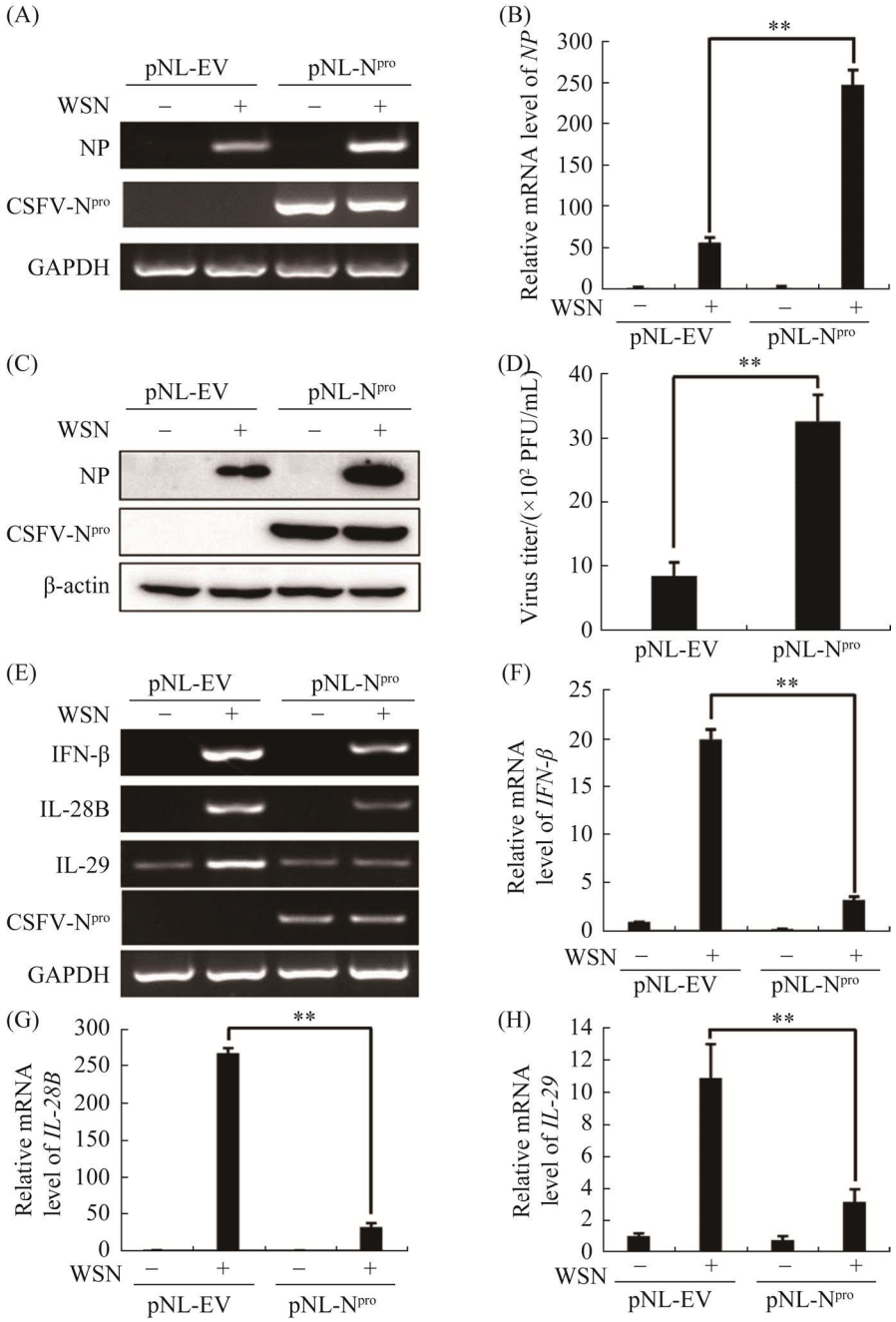

Figure 8 CSFV N^{pro} protein enhances IAV replication by suppressing type I and type III IFNs. A: pNL-N^{pro} or pNL-EV cells were infected with or without WSN virus, then the expression of *NP* mRNA was analyzed by RT-PCR. B: pNL-N^{pro} or pNL-EV cells were infected with or without WSN virus, then the *NP* mRNA was determined by RT-qPCR. C: pNL-N^{pro} or pNL-EV cells were infected with or without WSN virus, then the cell lysates were analyzed by Western blotting by using indicated antibodies. D: pNL-EV or pNL-N^{pro} cells were infected with or without WSN virus, then the cell supernatant was collected at 24h p.i. for titer determination by using plague assay. E: pNL-EV or pNL-N^{pro} cells were infected with or without WSN virus, then the indicated genes were analyzed by using RT-PCR. pNL-EV or pNL-N^{pro} cells were treated as described in (E), and then the the mRNA expression of *IFN-β* (F), *IL-28B* (G), and *IL-29* (H) was examined by RT-qPCR. Plotted are the average results from three independent experiments. **: $P<0.01$.

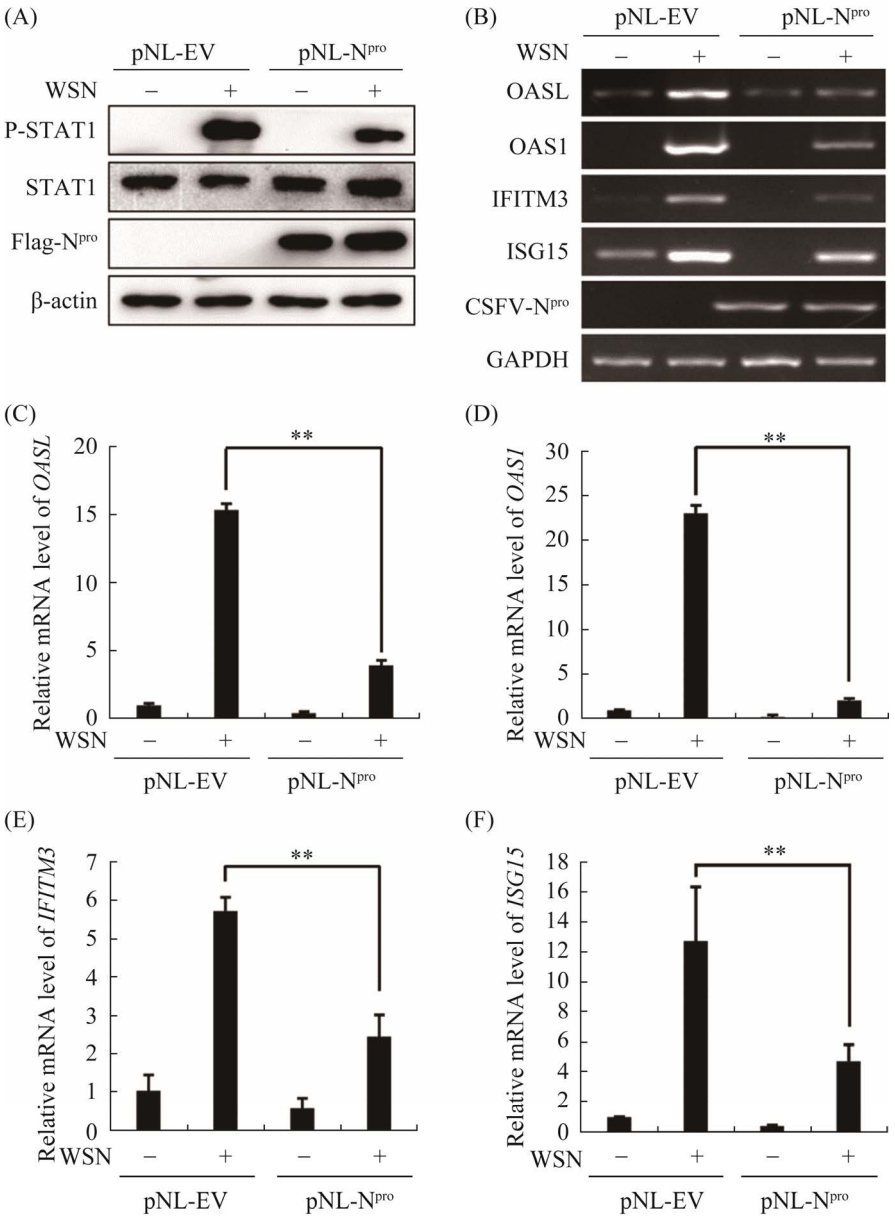

Figure 9 CSFV N^{pro} protein inhibits IAV-induced STAT1 phosphorylation and ISGs expression. A: pNL-EV or pNL-N^{pro} cells were infected with or without WSN virus, and then the cell lysates were analyzed by Western blotting using the indicated antibodies. B: pNL-EV or pNL-N^{pro} cells were infected with or without WSN virus, and then the expression of the indicated genes was examined by RT-PCR. pNL-EV or pNL-N^{pro} cells were treated as described in (B). Then the mRNA expression of *OASL* (C), *OAS1* (D), *IFITM3* (E), and *ISG15* (F) was examined by RT-qPCR. Plotted are the average results from three independent experiments. **: $P<0.01$.

Flaviviruses have evolved different strategies to antagonize IFN responses[57]. Hepatitis C virus (HCV) infection induces aberrant expression of two host microRNAs which can dampen host-antiviral responses by directly suppressing types I and type III IFN signaling pathway[58]. The expression of Type III IFNs is induced in bovine plasmacytoid dendritic

cells (pDC) infected with bovine viral diarrhea virus (BVDV)[59]. We have previously found that CSFV infection induces limited expression of type III IFNs compared to IAV infection, indicating that CSFV has established a strategy to suppress the expression of type III IFNs[28]. Here, we found that CSFV infection could suppress the expression of types I and type III IFNs induced by poly(I:C) and cytokines in PK-15 cells. It is known that type III IFNs are induced through similar signal transduction pathways as type I IFNs[60–61], and both type I and type III IFNs activate the JAK-STAT signaling pathway to achieve their antiviral function[62]. Therefore, we further determined whether CSFV could also inhibit the JAK-STAT signaling pathway. As expected, we found that CSFV infection resulted in a dramatic decline in the phosphorylation of STAT1 and the downregulation of *OASL*, *OAS1*, *IFITM3*, and *ISG15* induced by poly(I:C) and cytokines in PK-15 cells. Taken together, these findings suggest that CSFV dampens IFNs-mediated antiviral signaling to establish a successful infection in the host.

When the host cells are invaded by a virus, activation of pattern recognition receptors (PRRs) induces the production and release of type I and type III IFNs and other proinflammatory mediators to activate antiviral responses. Type I and type III IFNs bind to their unique receptors and mainly trigger the JAK/STAT signaling pathway to induce the expression of ISGs[63]. As a crucial component of JAK/STAT signaling, STAT1 is an obvious target for viral immune evasion[64]. Here, we found that CSFV infection inhibited the phosphorylation of STAT1, and suppressed the expression of *OASL*, *OAS1*, *IFITM3*, and *ISG15*, indicating that CSFV was able to directly suppress the activation of JAK-STAT signaling pathway mediated by cytokines in PK-15 cells.

N^{pro}, an important non-structural protein of CSFV, has protease activity and plays a key role in CSFV antagonism of host innate immunity. It is reported that CSFV N^{pro} protein can antagonize the host's natural immune response by interacting with host proteins, such as IRF3, IRF7, IκBα, and uS10[44]. Although great progresses have been made in the study of CSFV N^{pro} protein in antagonizing the natural immune response of the host, the role of CSFV N^{pro} protein in the natural immune response mediated by IFNs and the JAK-STAT signaling pathway has seldom been reported. Therefore, we explored the relationship between the CSFV N^{pro} protein and the natural immune response mediated by IFNs and the JAK-STAT signaling pathway by constructing PK-15 cell lines that stably overexpressed CSFV N^{pro} protein. Our study showed that CSFV could directly inhibit the JAK/STAT signaling pathway, and further observed that CSFV N^{pro} protein inhibited the phosphorylation of STAT1 and the expression of *OASL*, *OAS1*, *IFITM3*, and *ISG15* induced by poly(I:C) and cytokines.

Extensive studies on the co-infection or multiple infections of viral agents including CSFV, IAVs, porcine reproductive and respiratory syndrome virus (PRRSV), pseudorabies virus (PRV), porcine circovirus type 2 (PCV2), porcine rubulavirus, Japanese encephalitis virus, etc., in porcine have been largely performed in previous studies[21–26,53,65–67]. Co-infection of CSFV and IAVs is not uncommon in clinical practice, and poses continuous threats to pig industry[21]. According to an epidemiological survey in Guangxi province, two samples were proved to be co-infection of CSFV and IAVs among the 239 tissue samples of pigs with respiratory symptoms[24]. Triple or quadruple infection of IAVs with other pathogens, including CSFV, PRRSV, PCV-2, and PRV have been reported in clinical cases[22–23,25,27]. In addition, the co-infection of CSFV and AIVs can also cause secondary infection of bacterial diseases, resulting in more serious consequences[26–27]. To sum up, the mixed infection of CSFV and AIVs, together with other swine viruses, is relatively common in clinical cases and usually causes

heavy losses. Therefore, to clarify the mechanism of mixed infection of CSFV and IAVs is of great significance to prevention and control of viruses. However, the mechanism about the viral interactions, for instance, the influences of the first infectious agent on the secondary one is largely unknown. During IAV infection, the host antiviral response is activated and type I and type III IFNs are induced to antagonize viral infection[68–69]. In the present study, we found that CSFV N^{pro} protein could enhance the IAV replication through suppressing the expression of the type I and type III IFNs in swine cells. However, the clinical cases reported just indicates CSFV and IAV may be co-infected under natural conditions, while the promotion of replication of IAVs caused by CSFV *in vivo* still needs to be further studied.

In summary, the present study demonstrated that CSFV N^{pro} protein plays critical roles in the regulation of innate immunity. CSFV N^{pro} protein could suppress the expression of type I and type III IFNs. Importantly, further analysis revealed that N^{pro} protein was involved in the inhibition of the JAK-STAT signaling pathway activated by cytokines during CSFV infection, leading to a significantly declined expression of some critical ISGs. Moreover, we showed that CSFV N^{pro} protein could enhance the replication of IAV by suppressing type I and type III IFNs in PK-15 cells. Collectively, our data demonstrated that CSFV N^{pro} protein play significant roles in the inhibition of immune systems and might influence the secondary IAV infections.

Competing interest: the authors declare no conflict of interest.

Authors' contributions: WS, BC, J-LC, and SM designed the study. WS, BC, BY, HQ, FW, XC, YC, YL, and SM performed the experiments. WS, BC, J-LC, and SM analyzed the data and wrote the manuscript. SW and YC contributed to critical comments and revision of the manuscript. All authors meet the criteria for authorship. All authors have read and agreed to the published version of the manuscript.

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