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Classical swine fever virus non-structural proteins modulate Toll-like receptor signaling pathways in porcine monocyte-derived macrophages

Zhi Cao[a,b], Qian Yang[b], Minping Zheng[a], Huifang Lv[a], Kai Kang[a], Yanming Zhang[a,*]

[a] College of Veterinary Medicine, Northwest A&F University, Shaanxi, 712100, China
[b] Key Laboratory of Sustainable Development of Marine Fisheries, Ministry of Agriculture, Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Qingdao, 266071, China

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ABSTRACT

Toll-like receptors (TLRs) are crucial activators of the innate immune response that play various roles in viral infection. Studies have confirmed that classical swine fever virus (CSFV) infection has significant effects on the expression of immune effectors participating in TLR signaling pathways; however, the involvement of CSFV-encoded proteins in TLR signaling pathways remains unclear. In this study, lentiviral individually expressing CSFV non-structural proteins (NSPs) were constructed to identify the "key proteins" that affect TLR gene expression and to analyze the impacts of these proteins on factors downstream of the TLR signaling pathways. The results indicated that N^{pro}, NS2, NS3, NS3/4A, NS4B and NS5A all failed to induce the activation of NF-κB p65. Furthermore, NS4B was found to inhibit poly (I:C) stimulation-mediated activation of the TLR3 signaling pathway in porcine monocyte-derived macrophages (pMDMs), thereby suppressing the TRIF mRNA transcription, the IRF3 protein translation and the NF-κB p65 phosphorylation, and ultimately affecting the secretion of IL-6 and IFN-β; CSFV NS5A protein could significantly increase the activation of MyD88 and IRF7 as well as the consequent synthesis of IFN-α in pMDMs. The results suggest that CSFV NSPs affect TLR-mediated innate immune responses in pMDMs.

1. Introduction

Classical swine fever (CSF) is recognized as a serious swine disease that is characterized by hemorrhagic fever, disseminated intravascular coagulation, thrombocytopenia and immunosuppression (Lohse et al., 2012; Moennig, 2000). The aetiological agent of classical swine fever (CSF) is classical swine fever virus (CSFV), which belongs to the family *Flaviviridae* (Becher et al., 2003). The genome of CSFV is a single-stranded, positive-sense RNA molecule of approximately 12.3 kb that encodes a polyprotein, which yields four structural proteins (i.e. C, E^{rns}, E1 and E2) and eight mature non-structural proteins (NSPs) (i.e. N^{pro}, p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B) after processing. Currently, it is known that CSFV infection can inhibit apoptotic signaling (Johns et al., 2010) and is accompanied by immune suppression (Ganges et al., 2008), allowing CSFV to escape immune surveillance. However, the CSFV-encoded proteins that are involved in host-virus interactions are currently not fully understood.

Based on identifying characteristics, Toll-like receptors (TLRs) are closely linked to antiviral innate immunity (Lester and Li, 2014). TLR3, TLR7, TLR8 and TLR9 can detect viral nucleic acid substances in different forms (such as dsRNA, ssRNA and CpG DNA), thus initiating antiviral immune responses, while TLR2 and TLR4 are involved in the recognition of some viral-envelope proteins or viral components, resulting in the generation of inflammatory cytokines (Abe et al., 2012; Kawai and Akira, 2011; Moresco et al., 2011; Oliveira-Nascimento et al., 2012). The existing studies on CSFV-induced innate immunity have focused primarily on the activation of some immune cells (macrophages and plasmacytoid dendritic cells) as well as the secretion of type I IFNs and proinflammatory cytokines such as interleukin-6 (IL-6), IL-10, IL-12, and tumor necrosis factor (TNF)-α (Gladue et al., 2010; Hulst et al., 2012; Li et al., 2010). Also, the activation of the nuclear factor-kappa b (NF-κB) signaling pathway was inhibited by CSFV infection both in vitro and in vivo (Chen et al., 2012). It is well documented that the N^{pro} of CSFV interferes with alpha/beta interferon (IFN-α/β) synthesis by inducing proteasomal degradation of interferon regulatory factor 3 (IRF3), and interacts with IRF7 by limiting the IRF7-dependent IFN-α induction in SK-6 cells and pDC (Bauhofer et al., 2007; Fiebach et al., 2011). Furthermore, the transcriptional activation of TLR-7-induced genes is increased by the recombinant virus NS4B.VGIv infection (Fernandez-Sainz et al., 2010). Our previous study found that

* Corresponding author.
E-mail address: zhangym@nwsuaf.edu.cn (Y. Zhang).

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infection of porcine monocyte-derived macrophages (pMDMs) with the Shimen strain of CSFV could induce the expression of TLR2, TLR3, TLR4, TLR7 and type I IFNs, resulting in significant differences in the levels of proinflammatory cytokines (Cao et al., 2015). All these observations led to the hypothesis that CSFV-encoded proteins may have key functions in the TLRs-mediated innate immune response.

Additionally, another *Flaviviridae* family member, hepatitis C virus (HCV), affects the TLR-mediated innate immune response. The HCV NS3 induces the TNF-α and IL-10 secretion via the activation of TLR2 signaling pathways (Chang et al., 2007). The HCV proteins NS3/4A are involved in the degradation of TIR domain-containing adaptor inducing IFN-β (TRIF) and in the suppression of TLR3-mediated signaling pathways (Li et al., 2005b). HCV NS5A modulates the TLR signaling pathways through a direct interaction with MyD88 and ultimately affects the cytokine production (Abe et al., 2007); In addition, HCV NS3, NS3/4A, NS4B and NS5A can inhibit the TLR2, TLR4, TLR7 and TLR9 signal transduction pathways (Heim, 2013; Sato et al., 2007). The virus-encoded proteins of HCV and CSFV may have similarities in composition and function, which may provide further insight into the possible functions of the CSFV NSPs in affecting the TLR-mediated innate immune response.

Therefore, the purpose of this study is to determine the roles of swine TLRs in recognizing CSFV NSPs (N^{pro}, NS2, NS3, NS3/4A, NS4B, NS5A and NS5B) and to establish pMDMs expressing CSFV NSPs, which can identify the “strategic proteins” that affect TLR gene expression and can analyze the impact on factors downstream of the TLR signaling pathways.

2. Materials and methods

2.1. Cell culture

HEK-293T cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco, UK) with 10% heat-inactivated foetal calf serum (FCS) (Gibco, UK) and incubated at 37 °C with 5% CO_2.

Monocytes were isolated from the peripheral blood mononuclear cells (PBMCs) of pigs (all of which were negative for CSFV, porcine reproductive and respiratory syndrome virus and swine influenza virus antigens and antibodies) provided by Professor Zhi-Zhong Jing (Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Lanzhou, China) using Ficoll-Paque density (1.077 ± 0.001 g/ml) gradient centrifugation, as described previously (Cao et al., 2015). Isolated monocytes were plated at 1×10^6 cells/ml in RPMI 1640 medium (GE Healthcare, USA) supplemented with 20% heat-inactivated FCS for 8 days to obtain pMDMs.

2.2. Plasmid construction

DNA fragments encoding each of the CSFV NSPs were generated from a full-length cDNA clone of the Shimen strain (GenBank accession number AF092448) by PCR using PrimeSTAR® HS DNA polymerase (TaKaRa, China), and each gene was amplified with the primer pairs described in Table 1. To facilitate the cloning of the amplicons, *Eco*RI and *Bam*HI sites were engineered into the PCR oligonucleotides for amplification of the CSFV N^{pro}, NS2, NS4B, NS5A, and NS5B genes, while *Nhe*I and *Not*I sites were engineered into the PCR oligonucleotides for amplification of the CSFV NS3 NS3/4A genes. Moreover, a FLAG tag (GATTACAAGGATGACGACGATAAG) was inserted at the 3’ end of the sequence of each CSFV NSP. The PCR products were subcloned into the lentivector pCDH-CMV-MCS-EF1-GFP/Puro. The recombinant plasmids were restriction enzyme-digested, the correct band size was identified, and the samples were sent for DNA sequencing. The recombinant plasmids were named pCD513B-N^{pro}-Flag, pCD513B-NS2-Flag, pCD513B-NS3-Flag, pCD513B-NS3/4A-Flag, pCD513B-NS4B-Flag, pCD513B-NS5A-Flag and pCD513B-NS5B-Flag.

2.3. Lentiviral packaging of the CSFV NSPs

HEK-293T cells were seeded into 6-well plates at a concentration of 1×10^6 cells/well. Using X-tremeGENE HP DNA transfection reagent (Roche, Switzerland), the recombinant plasmids and control plasmid (pCDH-CMV-MCS-EF1-GFP/Puro) were individually transfected into overnight-cultured cells (grown to 70–80% confluency). Briefly, 1.5 μg of plasmid packaging mix (GAG/pol, Rev and VSV-G, 0.5 μg each) and 0.5 μg recombinant plasmid were added to 100 μL Opti-MEM culture medium (Gibco, UK) followed by the addition of transfection reagent. The complexes were mixed gently at room temperature (r.t.) and incubated for 15 min. Then, the transfection complex was added dropwise to the cell culture supernatant. After 24 h, the medium was replaced with Advanced DMEM (Gibco, UK) containing 20 mL/L FCS, 4.0 mM L-glutamine (Invitrogen, USA), 0.01 mmol/L cholesterol (Sigma, USA) and 1:1000 diluted Chemically defined lipid (Invitrogen, USA). After a 48-h culture, the cell supernatants were collected. After centrifugation at $1500 \times g$ for 15 min, the supernatants, containing the lentiviruses expressing the CSFV NSPs and control lentivector, were collected.

2.4. Lentivirus infection

The number of GFP-positive cells was determined by fluorescence microscopy (Nikon, Japan) to calculate the virus titre (TU/mL), which is the average number of GFP-positive cells (C) multiplied by the dilution factor (D) divided by the virus inoculation volume (V).

After trypsinization, pMDMs were seeded into 12-well plates at a density of 5×10^5 cells/well and grown to 70%–80% confluency. The medium was then replaced with RPMI 1640 containing 10% FBS and 8 μg/μL polybrene. Then, the cells were infected with lentiviruses encoding the CSFV NSPs and control lentivector by adding 10^7 TU/mL of virus to each well, with phosphate-buffered saline (PBS) as a control. After a 24-h incubation, the inoculum was discarded, and the cells were washed with PBS, pH 7.4, and then incubated in RPMI 1640 medium containing 5% FBS and 6 μg/μL puromycin. At 48 h post-transfection (hpi), the cell-free culture supernatants and cell lysates were harvested and stored at −80 °C until use.

2.5. Western blot analysis

Cell lysates were prepared from control cells, cells infected with lentiviruses encoding the CSFV NSPs and control lentivector for 48 h, respectively, and cells were seeded into 12-well plates at a concentration of 5×10^5 cells per well and infected with lentiviruses encoding the CSFV NSPs and control lentivector for 24 h, then stimulated with TLR-specific ligand [poly (I:C) (Sigma, USA), which is ligand for TLR3] or with CSFV Shimen strain for 24 h. Next, the cytoplasmic and nuclear proteins were isolated using a nuclear and cytoplasmic protein extraction kit (Thermo, USA). Firstly, the translation of the CSFV NSPs was detected in total cell extracts of each cell by western blot analysis using a FLAG-tag antibody (Sigma, USA). Secondly, the effects of the CSFV NSPs on the protein levels of TLR2, TLR3, TLR4, TLR7, IRF3, IRF7 and IκBα in total cell extracts and on the phosphorylation status of NF-κB p65 and pERK1/2 in nuclear extracts were analyzed by western blot, as described previously (Cao et al., 2015). Briefly, the processed protein sample was subjected to protein electrophoresis, and after completion, the protein was transferred from the gel to the polyvinylidene fluoride (PVDF) membrane under the action of a constant pressure of 15 V. The PVDF membrane was placed in blocking solution (TaKaRa, China) and incubated for 2 h at r.t. with shaking. PBS with Tween 20 (PBST) containing an appropriate amount of each protein primary antibody and incubated overnight at 4 °C. After the primary antibody was incubated, it was rinsed 4 times with PBST for 10 min each time. Then, it was incubated in PBST containing the corresponding secondary antibody for 2 h at r.t., after which it was rinsed 4 times with PBST for 10 min each time. Finally, exposure was carried out using the HRP-ECL

Table 1
Construction of CSFV non-structural protein subcloned and primer sets used in this study.

Genes	Primer (5'–3')	Restriction sites
N^{pro}-F	CGGAATTCATGGAGTTGAATCATTTTG	*Eco*RI、*Bam*HI
N^{pro}-R	CCGGATCCTTACTTATCGTCGTCATCCTTGTAATCGCAACTGGTAACCC	
NS2-F	CGGAATTCATGGGAAAGATAGATGG	
NS2-R	CCGGATCCTTACTTATCGTCGTCATCCTTGTAATCTCTAAGCACCCAGCC	
NS3-F	CTAGCTAGCATGGGGCCTGCCGTTTGC	*Nhe*I、*Not*I
NS3-R	ATAAGAATGCGGCCGCTTACTTATCGTCGTCATCCTTGTAATCTAGACCAACTACTTG	
NS3/4A-F	CTAGCTAGCATGGGGCCTGCCGTTTGC	
NS3/4A-R	ATAAGAATGCGGCCGCTTACTTATCGTCGTCATCCTTGTAATCTAGCTCCTTCAATTC	
NS4B-F	CGGAATTCATGGCTCAGGGGGATGTG	*Eco*RI、*Bam*HI
NS4B-R	CCGGATCCTTACTTATCGTCGTCATCCTTGTAATCTAGCTGGCGGATCTTC	
NS5A-F	CGGAATTCATGTCAAGTAATTACATTC	
NS5A-R	CCGGATCCTTACTTATCGTCGTCATCCTTGTAATCCAGTTTCATAGAATAC	
NS5B-F	CGGAATTCATGAGTAATTGGGTGATG	
NS5B-R	CCGGATCCTTACTTATCGTCGTCATCCTTGTAATCTACCCCTCTCCCTATC	

luminescence method: A and B luminescent liquids were mixed in equal proportions, dropped onto the film, and analyzed using a gel imaging system.

2.6. Quantitative real-time PCR (qrt-PCR) analysis

The relative mRNA expression of the TLR2, TLR3, TLR4 and TLR7 genes and of MyD88, TRIF, IRF3, IRF7, NF-κB p65 and IκBα, which are key elements of TLR signal transduction pathways, were determined by qrt-PCR analysis, and each gene was amplified with the primer pairs described in Table 2 (Cao et al., 2015). Total RNA was prepared from cell lysates infected with NSPs-expressing lentivirus for 48 h and the NSPs-expressing lentiviruses-infected pMDMs (24 h) were stimulated for 24 h with appropriate content of poly (I:C), using an RNeasy Mini kit (Qiagen, Germany).

First-strand cDNA was synthesized using a PrimeScript™ reverse transcription (RT) reagent kit with gDNA Eraser (TaKaRa, China). qrt-PCR was performed using a Bio-Rad iQ5 system (Bio-Rad, CA, USA) and SYBR Premix Ex Taq II (TaKaRa, China) with the following program: 95 °C for 5 s followed by 40 cycles of 95 °C for 5 s and 60 °C for 20 s. The expression of mRNAs of each of the key elements of TLR signal transduction pathways, and TLR was normalized with that of β-actin mRNA.

2.7. RT-PCR analysis

Total RNA was prepared from the cell lysates of "Lentivirus infection" using an RNeasy Mini kit (Qiagen, Germany). First-strand cDNA was synthesized using a PrimeScriptTM RT reagent kit with gDNA Eraser (TaKaRa, China). The following primer set was designed based on the sequence of the multiple cloning site of the pCDH-CMV-MCS-EF1-GFP/Puro vector to verify the presence of target gene of interest in pMDMs: (Forward: 5'-3') GACCTCCATAGAAGAT and (Reverse: 5'–3') GGCGATGTGCGCTCTG. The PCR reactions were performed in a total volume of 50 μL, containing 1 μL Ex Taq (5 U/μL), 5 μL 10 × Ex Taq buffer, 4 μL dNTP mixture, 10 ng cDNA template, 0.5 μL upstream primer (20 pmol/μL), 0.5 μL downstream primer (20 pmol/μL), and sterile H_2O to bring the volume up to 50 μL. The reaction conditions were as follows: an initial denaturation at 94 °C for 1 min; 30 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s, and elongation at 72 °C for 2 min; a final extension at 72 °C for 8 min; and 4 °C upon completion of the reaction.

Table 2
Host genes analyzed and primer sets used in this study.

Genes	Forward primer (5'–3')	Reverse primer (5'–3')
β-actin	CAAGGACCTCTACGCCAACAC	TGGAGGCGCGATGATCTT
TLR2	GGAGCCTTAGAAGTAGAGTTTG	TGTATCCACATTACCGAGGG
TLR3	TAACAACCTTCCAGGCATA	AAGAGGAGAATCAGCGAGTG
TLR4	TCTACATCAAGTGCCCCTAC	ATTCTCCCAAAACCAAC
TLR7	CATGTGATCGTGGACTGC	GTGATGCTCGCTATGTGG
MyD88	GCCGTCGGATGGTAGTGGTT	TGGTGCAGGGGTTGGTGTAG
TRIF	TGGGACATCCTTAGGGACATG	CCAGTGGACCTCAGGGAATG
IRF3	CATAACTGGTGGTGGTGATGAC	TCTGCTCTGCTTGTGGTTGA
IRF7	TGCGATGGCTGGATGAAG	AGGGCACAGCGGAAGTTG
NF-κB p65	GGGACTACGACCTGAATGCT	GGGCACGGTTGTCAAAGAT
IκBα	GAACGTGAACGTCCTCTG	TCATCATATTAGTGCCCTGT

2.8. Enzyme-linked immunosorbent assay (ELISA) analysis

Cells were seeded into 24-well plates at a concentration of 4×10^5 cells per well and infected with lentiviruses encoding the CSFV NSPs and control lentivector for 24 h, then stimulated with the ligand poly (I:C) or with CSFV Shimen strain for 24 h. Cell culture supernatants were collected and analyzed using a Duoset ELISA Development System for porcine IL-1β and IL-6 (R&D Systems, USA). The secreted protein levels of IFN-α and IFN-β were determined with IFN-α antibody (G16) and IFN-β antibody (LSBio, USA), respectively. All stimulations were performed according to the manufacturer's instructions, and all samples were tested in triplicate and read at 450 nm using an ELISA plate reader (Thermo, USA).

2.9. Statistical analysis

Data are shown as the means ± standard deviation (SD) of 3 independent experiments and were analyzed by SPSS 17.0 software. A p value less than 0.05 was considered significant and was calculated using Student's t-test.

3. Results

3.1. pMDMs successfully expressed the CSFV NSPs

We first constructed a lentiviral vector expressing CSFV NSPs, and packaged with lentivirus carrying CSFV NSPs in HEK-293T cells, and then used lentivirus with CSFV NSPs to infect pMDMs. The GFP-positive fluorescent cells were examined by RT-PCR (Fig. 1A) and western blot analysis using a FLAG-tag antibody (Fig. 1B). Screened samples of cells which can successfully expressed each NSP were subjected to downstream assays.

3.2. The expression of TLR2, 3, 4, and 7 influenced by CSFV NSPs

To determine which of the CSFV NSPs is a "key protein" influencing the expression of TLR2, TLR3, TLR4 and TLR7, pMDMs were transiently infected with CSFV NSP-expressing lentiviruses for 48 h, and qrt-PCR

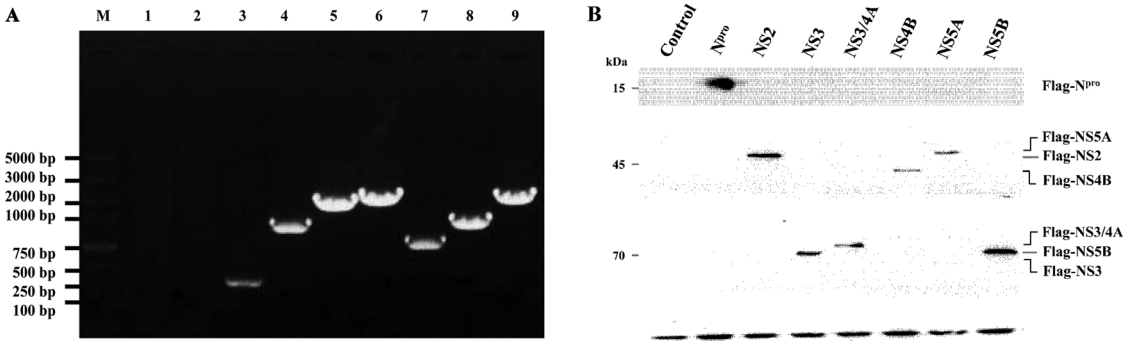

Fig. 1. Identification of pMDMs expressing the CSFV NSPs. **(A)** The nucleic acid expression levels of the CSFV NSPs, as analyzed by RT-PCR. Lane M is DL5000 DNA Marker; lane 1 is the negative control; lane 2 is the control lentivector; lanes 3–9 are N^{pro} (504 bp), NS2 (1371 bp), NS3 (2049 bp), NS3/4A (2241 bp), NS4B (1041 bp), NS5A (1491 bp), NS5B (2154 bp), respectively. **(B)** The expression of the FLAG-tagged CSFV NSPs, as analyzed by western blot. Cell lysates were prepared from pMDMs expressing each of the CSFV N^{pro}, NS2, NS3, NS3/4A, NS4B, NS5A and NS5B and immunoblotted with antibodies against the FLAG-tagged.

analysis was used to detect the expression of TLR2, TLR3, TLR4 and TLR7. As shown in Fig. 2A, TLR2 showed an increased expression pattern in cells infected with lentiviruses expressing NS2 and NS3, the expression of TLR4 was significantly enhanced by NS2, while the expression of TLR3 was slightly reduced by NS3 and NS3/4A but was significantly decreased by NS4B. Moreover, TLR7 expression, was significantly increased by NS2, NS3/4A and NS5A but was slightly reduced by NS4B. However, infection of cells with lentiviruses expressing NS5B had no effect on the expressions of TLR2, TLR3, TLR4 and TLR7. Therefore, in the following studies, we focused on N^{pro}, NS2, NS3, NS3/4A, NS4B and NS5A.

To further substantiate these results, we verified the level of protein expression by western blot analysis. As shown in Fig. 2B, most results were in accordance with the qrt-PCR assays. However, according to the results of western blot analysis, NS4B could only slightly reduce the expression of TLR3 at an insignificant level. Additionally, insignificant up-regulation were also observed in the expression of TLR2 and TLR7 influenced by NS3, NS2 and NS3/4A, respectively.

3.3. CSFV NSPs affect the key proteins of the TLR signaling pathways

The above results identified the CSFV NSPs that could activate or inhibit TLRs, but whether these proteins affect TLR-mediated signaling pathways required further study. Therefore, we focused on investigating the key proteins in the TLR signaling pathways at both the mRNA and protein level in pMDMs expressing the CSFV NSPs via lentiviral infection. qrt-PCR results showed that the expression of MyD88 was significantly enhanced in pMDMs infected with CSFV NS2, NS3 and NS5A in comparison with control lentivector-expressing lentiviruses (CMV), respectively; Meanwhile, the transcription levels of IRF7 were significantly up-regulated after incubation with CSFV NS2, NS3/4A and NS5A-expressing lentiviruses, while TRIF gene expression was inhibited in pMDMs infected with NS4B-expressing lentiviruses (Fig. 3A). Furthermore, IRF3, NF-κB p65 and IκBα expression were not significantly influenced in either group (Fig. 3A).

Using western blot assays (Fig. 3B), we demonstrated that the protein expressions of IκBα appeared to be correlated with the NSP-mediated effects on the mRNA expression, and the phosphorylation level of NF-κB p65 indicated that none of the NSPs (N^{pro}, NS2, NS3, NS3/4A, NS4B and NS5A) could activate NF-κB p65. However, compared with the mRNA expression, NS2 and NS3/4A resulted in a slight increase in IRF7 protein levels respectively, and the expression of IRF3 was significantly decreased by N^{pro} and NS4B at the protein level. Additionally, the phosphorylation of ERK1/2 was significantly increased by NS3.

3.4. CSFV NSPs affect the immune effector molecules downstream of the TLR signaling pathways

In view of the above results, it was necessary to determine whether the CSFV NSPs affected the major immune effector molecules downstream of the TLRs. The protein levels of IL-1β, IL-6, IFN-α and IFN-β in the culture supernatants of cells infected with the CSFV NSP-expressing lentiviruses for 48 h were analyzed using ELISA assays. As shown in Fig. 4, the generation of IFN-α was significantly enhanced by NS5A but showed a decreasing trend in response to N^{pro}; the production of IL-6 and IFN-β were reduced to some extent by NS4B. Meanwhile, N^{pro} significantly inhibited IFN-β production, and NS2 and NS3 could promote the secretion of IL-1β.

3.5. CSFV NS4B inhibits the TLR-TRIF-dependent signaling pathway

To determine whether NS4B inhibit the TLR3-mediated innate immune response, after pMDMs was infected by lentivirus expressing CSFV NS4B, control plasmid or CSFV Shimen strain for 24 h, the expression of TRIF, IRF3, IL-6 and IFN-β in the cells was detected after 24 h of stimulation with the ligand poly (I:C) of TLR 3. As observed in Fig. 5A, with the stimulation of the ligand poly (I:C), cells transiently expressing NS4B exhibited significant inhibition of TRIF expression and slightly down-regulated IRF3 expression, comparing to the empty vector mock cells. From Fig. 5B and C, under the stimulation of poly (I:C), the NS4B expression in pMDMs inhibited the expression of IRF3 protein, the phosphorylation level of NF-κB p65, as well as the production of IFN-β and IL-6.

4. Discussion

Toll-like receptors (TLRs) have been reported to activate NF-κB, mitogen-activated protein kinases (MAPKs) and interferon regulatory factors (IRFs) via the MyD88-dependent and TRIF-dependent pathways to induce pro-inflammatory cytokines and type I and/or type III IFNs, and to set up a bridge of communication subsequent to the onset of adaptive immunity (Akira et al., 2006; Bowie and Unterholzner, 2008; Kawai and Akira, 2010). At present, the relationship between CSFV infection and TLRs-mediated innate immune response is still in the preliminary stage. A comprehensive and systematic study of the molecular interaction between CSFV NSPs and TLRs has not been reported. This study aimed to further identify the key CSFV nonstructural proteins that affect TLR expression and analyze the impact of these proteins on factors downstream of TLR signaling.

It has been well known over several years that the IRF3 protein is degraded by N^{pro} (Bauhofer et al., 2007; La Rocca et al., 2005), and this study found that CSFV N^{pro} protein could degrade it in pMDMs as well. Furthermore, it is well known that TLR3 mediates the activation of

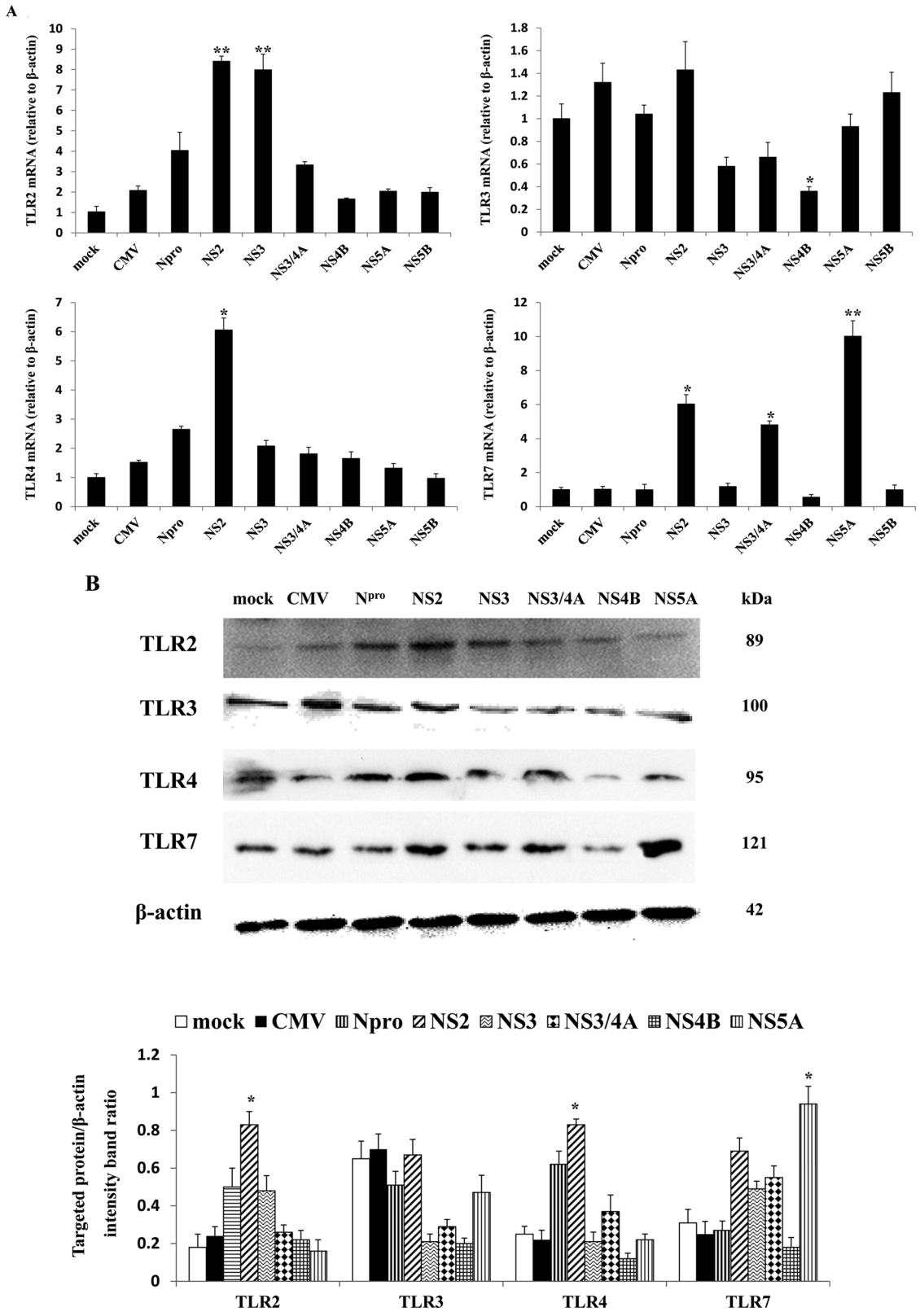

Fig. 2. Effects of CSFV NSPs on the expression of TLR2, 3, 4, and 7. **(A)** pMDMs were transiently infected with CSFV NSP-expressing lentiviruses and control lentivector-expressing lentiviruses (CMV) for 48 h. Total RNA from the infected (CSFV NSP-infected and CMV-infected) and uninfected cells (mock) was analyzed by qrt-PCR at 48 hpi. **(B)** Western blot analysis of the expression of TLR2, 3, 4, and 7 in total cellular extracts of pMDMs that were transiently infected with CSFV NSP-expressing lentiviruses (except NS5B) and control lentivector-expressing lentiviruses (CMV) for 48 h. β-actin served as an internal control. The relative levels of the targeted proteins were showed by histograms representing density readings of the gel bands and the ratios were calculated relative to the β-actin control. The data represent the mean ± SD of 3 independent experiments. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$, calculated using Student's *t*-test in NSP-expressing lentiviruses-infected vs. CMV-infected groups for the corresponding hpi.

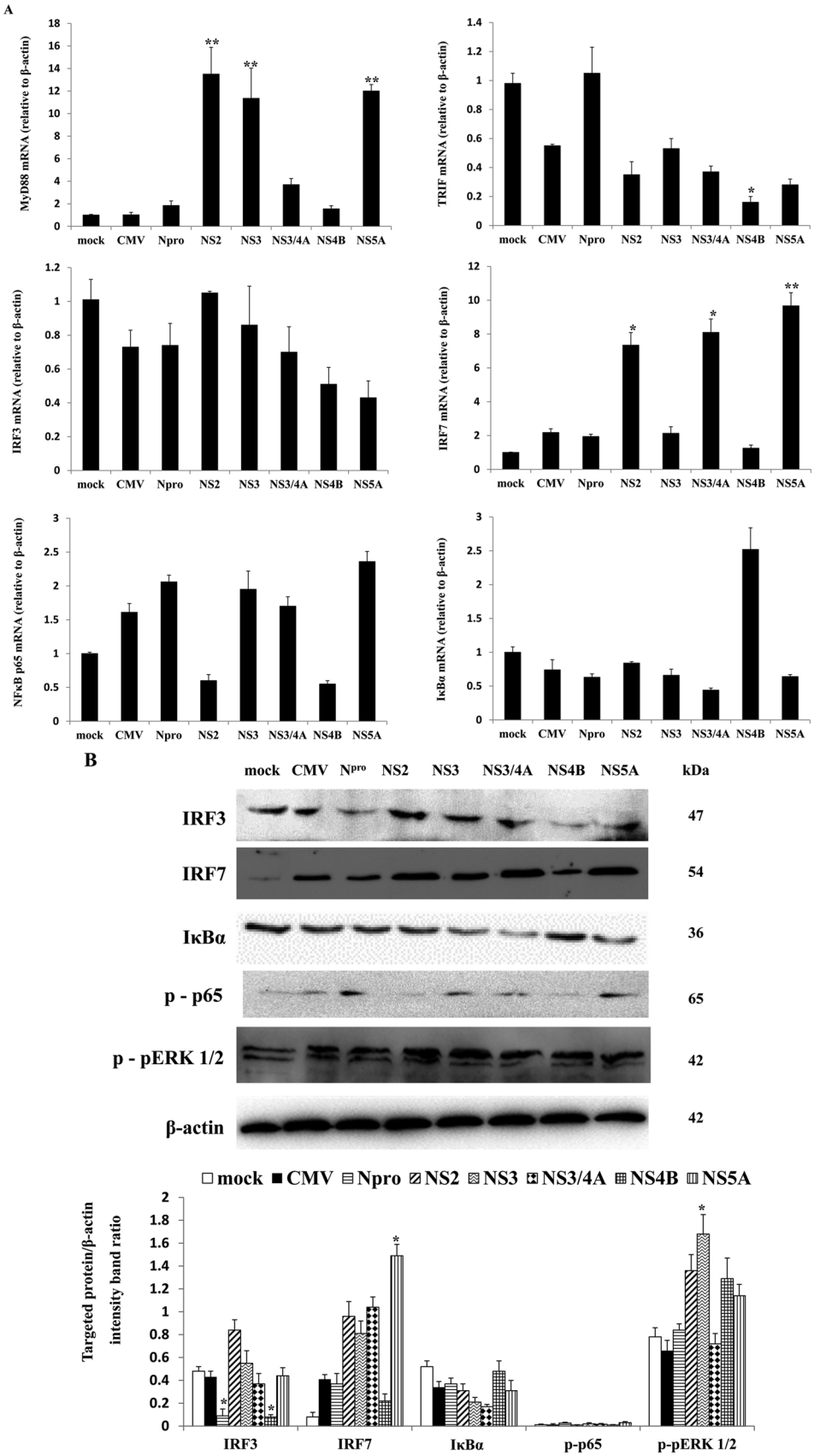

Fig. 3. The effects of the CSFV NSPs on the expression of key proteins in the TLR signaling pathways. **(A)** The effects of the CSFV NSPs on the mRNA expression of MyD88, TRIF, IRF3, IRF7, NF-κB p65 and IκBα. pMDMs were transiently infected with CSFV NSP-expressing lentiviruses (except NS5B) and control lentivector-expressing lentiviruses (CMV) for 48 h. Total RNA from the infected (CSFV NSP-expressing lentiviruses and CMV-infected) and uninfected cells (mock) was analyzed at 48 hpi. **(B)** Western blotting analysis of total cellular extracts for IRF3, IRF7 and IκBα, and the phosphorylation status of ERK1/2 and NF-κB p65 in pMDMs infected with CSFV NSP-expressing lentiviruses (except NS5B) in comparison with CMV-infected cells. β-actin served as an internal control. The relative levels of the targeted proteins were showed by histograms representing density readings of the gel bands and the ratios were calculated relative to the β-actin control. The data represent the mean ± SD of 3 independent experiments. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$, calculated using Student's *t*-test in NSP-expressing lentiviruses-infected vs. CMV-infected groups for the corresponding hpi.

downstream IRF3 and NF-κB pathways by recruiting the adaptor protein TRIF (Alexopoulou et al., 2001; Li et al., 2005a). Our most important finding was that NS4B could significantly inhibit the expression of key adaptor proteins (such as TRIF, IRF3 and NF-κB) and their downstream immune effector molecules (IFN-β and IL-6) in TLR3 signaling pathway stimulated by poly (I:C), and these proteins were

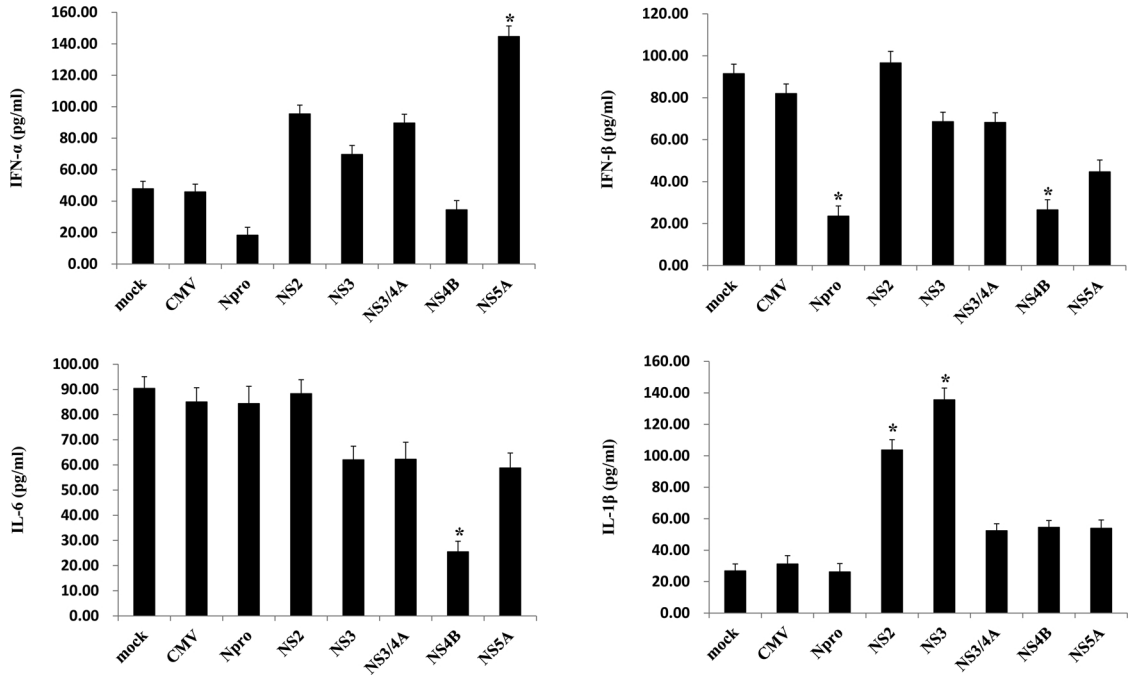

Fig. 4. The effects of the CSFV NSPs on the productions of IL-1β, IL-6, IFN-α and IFN-β. pMDMs were transiently infected with CSFV NSP-expressing lentiviruses (except NS5B) and control lentivector-expressing lentiviruses (CMV). After a 48-h incubation, the cell culture supernatants were collected and analyzed by ELISA. Data are shown as the means ± SD of 3 independent experiments.

expressed in a way that were relevant to CSFV infection. Thus, the NS4B may participate in disrupting TLR3-TRIF signaling pathways in pMDMs, which is similar with the degradation of TRIF to inhibit TLR3-mediated antiviral interferon expression pathway by the HCV NS4B protein (Liang et al., 2018).

After recognition of ssRNA, TLR7 initiated a MyD88-dependent pathway, leading to the activation of IRF7 and the consequent synthesis of type I interferon (Crozat and Beutler, 2004). In HCV, the expression of the NS5A protein has been reported to inhibit TLR-MyD88-induced signaling in macrophage cells (Abe et al., 2007). However, controversial results were observed in the present study showed that CSFV NS5A protein or the Shimen strain of CSFV were involved in upregulating the transcription of the MyD88, and in enhancing the activation of IRF7 as well as the consequent synthesis of IFN-α in the pMDMs. Therefore, it is possible that the expression of NS4B protein may disrupt TLR-TRIF, while NS5A probably activates TLR-MyD88 signaling pathways in the pMDMs. However, the mechanism by which NS4B and NS5A inhibit or activate the TLR signaling pathway in the pMDMs still needs further investigation.

In the present study, our data with pMDMs infected with NS4B-expressing lentiviruses showed that IRF3 was down-regulated at the protein level, since only minor down-regulation were found in the IRF3 mRNA quantities, which is consistent with the previous observation that N^{pro} could not down-regulate IRF3 expression but promote proteasomal degradation of IRF3 (Bauhofer et al., 2007). Unfortunately, we could not find suitable antibodies for the detection of porcine MyD88 and TRIF, and were thus only able to analyze their expressions at the transcriptional level. Further study is needed to design overexpression and shRNA interference vectors for MyD88 and TRIF, and to express the CSFV NS4B or NS5A protein stably using the macrophage cell lines.

TLR signaling pathways are reported to induce the secretion of inflammatory cytokines through activation of the transcription factor NF-κB and MAPKs (Warner and Nunez, 2013). Here, we observed that the CSFV NSPs failed to induce the phosphorylation of NF-κB p65, which corresponded with the previous opinion that CSFV could not activate the NF-κB signaling pathway (Chen et al., 2012; Doceul et al., 2008). Moreover, the phosphorylation of ERK1/2, a MAPK signaling pathway, was significantly activated by NS3 and slightly activated by NS2, NS4B and NS5A, and was complementary to CSFV infection. Therefore, CSFV could stimulate the secretion of cytokines, such as IL-1β, probably caused by activation of MAPKs signaling cascade.

In this study, we determined the effects of CSFV NSPs on the TLR-mediated innate immune response by investigating the role of these proteins in the regulation of TLRs and their downstream signaling cascade. The results showed that the expression of NS4B resulted in significant suppression of TRIF, IRF3 and NF-κB p65, thereby inhibiting the secretion of IFN-β and IL-6 upon stimulation with poly (I:C). Furthermore, the NS5A protein was shown to enhance the activation of MyD88 and IRF7 as well as the subsequent IFN-α in pMDMs. These findings may be explored to provide a better understanding of the escape of CSFV from the host immune surveillance system.

Author contributions

Formal analysis, Minping Zheng and Huifang Lv; Funding acquisition, Yanming Zhang; Investigation, Kai Kang; Methodology, Zhi Cao, Minping Zheng and Huifang Lv; Project administration, Yanming Zhang; Writing – original draft, Zhi Cao; Writing – review & editing, Zhi Cao and Qian Yang.

Conflicts of interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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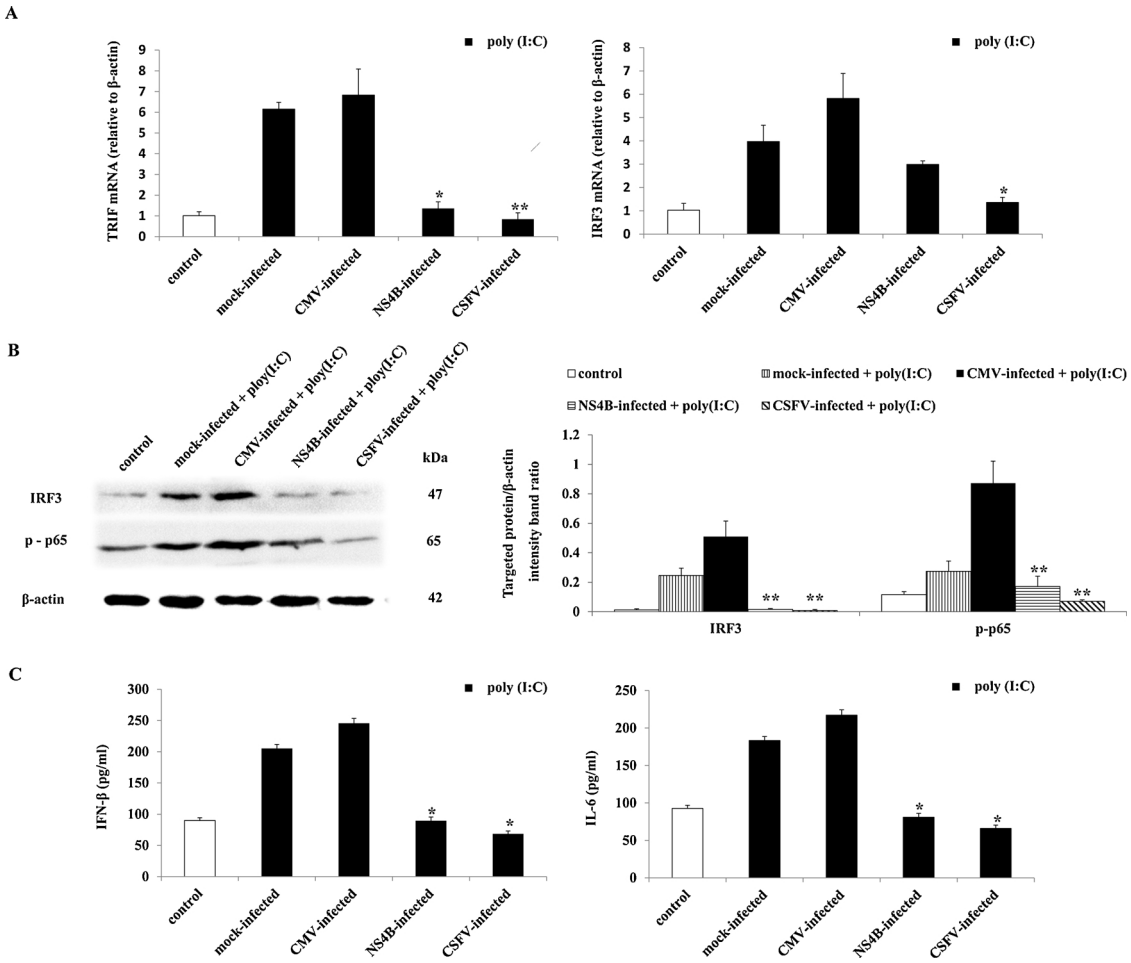

Fig. 5. CSFV NS4B modulates the TLR-TRIF-dependent signaling pathway in pMDMs. NS4B-infected, CSFV (Shimen-strain)-infected, CMV (control lentivector expressing lentiviruses)-infected or mock-infected pMDMs (24 h) were stimulated for 24 h with 10 mg poly (I:C) ml^{-1}. **(A)** TRIF and IRF3 mRNA expression levels in NS4B-infected, CSFV-infected, CMV-infected or mock-infected pMDMs either stimulated with poly (I:C) or untreated (control) were determined by qrt-PCR. **(B)** Western blotting analysis of total cellular extracts for IRF3 and the phosphorylation status of NF-kB p65 in NS4B-infected, CSFV-infected, CMV-infected or mock-infected pMDMs either stimulated with poly (I:C) or untreated (control). β-actin served as an internal control. The relative levels of the targeted proteins were showed by histograms representing density readings of the gel bands and the ratios were calculated relative to the β-actin control. **(C)** The production of IL-6 and IFN-β in culture supernatants of NS4B-infected, CSFV-infected, CMV-infected or mock-infected pMDMs either stimulated with poly (I:C) or untreated (control) were measured by ELISA. Data are shown as the mean ± SD of 3 independent experiments. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$, calculated using Student's *t*-test in NS4B-infected or CSFV-infected vs. CMV-infected groups for the corresponding hpi.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2019.01.025.

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