A comparison of the impact of Shimen and C strains of classical swine fever virus on Toll-like receptor expression

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Classical swine fever is one of the most important swine diseases worldwide and has tremendous socioeconomic impact. In this study, we focused on the signalling pathways of Toll-like receptors (TLRs) because of their roles in the detection and response to viral infections. To this end, two classical swine fever virus (CSFV) strains, namely the highly virulent CSFV Shimen strain and the avirulent C strain (a vaccine strain), were employed, and the expression of 19 immune effector genes was analysed by real-time PCR, Western blot analyses, ELISA and flow cytometry analyses. In vitro experiments were conducted with porcine monocyte-derived macrophages (pMDMs). The results showed that the mRNA and protein levels of TLR2, TLR4 and TLR7 were upregulated in response to CSFV infection, but TLR3 remained unchanged, and was downregulated after infection with the C strain and the Shimen virus, respectively. Furthermore, TLR3-mediated innate immune responses were inhibited in Shimen-strain-infected pMDMs by stimulation with poly(I : C). Accordingly, comprehensive analyses were performed to detect TLR-dependent cytokine responses and the activation of TLR signalling elements. CSFV infection induced mitogen-activated protein kinase activation, but did not elicit NFκB activation, thereby affecting the production of pro-inflammatory cytokines. The Shimen strain infection resulted in a significant activation of IFN regulatory factor IRF7 and suppression of IRF3. These data provided clues for understanding the effect of CSFV infection on the TLR-mediated innate immune response and associated pathological changes.

INTRODUCTION

Classical swine fever, which is caused by virulent strains of classical swine fever virus (CSFV), is still a widespread disease in developing countries, such as China, and has caused tremendous losses in the swine industry (Edwards et al., 2000; Luo et al., 2014). CSFV is a small, enveloped virus with a positive-sense ssRNA molecule of ~12.3 kb in length encoding a polyprotein and is classified as a member of the genus Pestivirus within the family Flaviviridae (Becher et al., 2003). Like all pestiviruses, the virulent strains of CSFV can escape immune surveillance and establish persistent infection (Moennig, 2000). The C strain of CSFV, which is attenuated from a highly virulent strain (possibly the Shimen strain), is able to induce sterile immunity and provides complete protection against different genotypes of CSFV (Luo et al., 2014). Sequence alignment shows that the 3’ UTR of the C strain has a 12 nt insertion (CUUUUUCUUUU), which is not present in other CSFV strains, leading to attenuation of the Shimen strain (Wang et al., 2008). Molecular mechanisms of innate immune response to the Shimen and C strains of CSFV remain poorly understood.

Toll-like receptors (TLRs) are the fundamental sensor molecules of the host innate immune system. Through sensing the conserved structures of viruses, these receptors initiate innate immune responses via distinct signalling pathways (Kawai & Akira, 2010). To date, 13 TLRs have been identified and characterized for their particular cognate ligands (Kawai & Akira, 2010). For example, TLR2 and TLR4 recognize the virus envelope protein (Lester & Li, 2014); TLR7 and TLR8 have been shown to detect GU-rich and AU-rich ssRNA sequences of RNA viruses (Heil et al., 2004); and TLR3 is able to sense dsRNA viruses, ssRNA viruses and DNA viruses (Kawai & Akira, 2011). Relevant studies on innate immunity induced by CSFV have mainly focused on the activation of immune cells and secretion of type I IFNs and pro-inflammatory cytokines (Borca et al., 2008; Gladue et al., 2010; Li et al., 2010). However, CSFV may also inhibit important proteins in the antiviral immune response pathway (Bauhofer et al., 2007; Fiebach et al., 2011; Luo et al., 2009). Unfortunately,
data on the molecular interactions between CSFV and TLRs are scarce.

Therefore, the objective of the present study was to analyse the induction of differential gene expression in TLR pathways by the Shimen and C strains of CSFV in porcine monocyte-derived macrophages (pMDMs) to reveal specific mechanisms of the host innate immune response to the CSFV Shimen strain relative to those of the C strain.

RESULTS

Confirmation of CSFV infection in pMDMs

Viral loads in pMDMs that were collected at various times after infection were determined by real-time (RT)-PCR as described in Methods. CSFV RNA was detected 24, 48 and 72 h after inoculation, and the results indicated that the pMDMs were successfully infected with CSFV, both the Shimen and C strains (Fig. 1). The amount of viral RNA increased with time, with the highest titres observed after 72 h. CSFV nucleic acid was not detected in the mock-inoculated pMDMs at any of the experimental time points.

TLR gene expression in response to CSFV Shimen and C strain infection

To examine the expression levels of all of the known TLRs during CSFV infection, we first performed RT-PCR on the TLR-specific mRNAs in pMDMs infected with either the Shimen or C strain using primers specific for each of the 10 known TLRs. Most of the TLRs were expressed at the same levels (e.g. TLR1, TLR5, TLR6, TLR8 and TLR9) except for TLR10 (undetectable) in both the Shimen- and C-strain-infected cells (date not shown). Interestingly, the expression levels of TLR2, TLR4 and TLR7 were upregulated (by both CSFV strains), but TLR3 was downregulated by the Shimen strain (the C-strain–infected cells showed no obvious TLR3 decrease) at the three time points examined (Fig. 2a).

We also examined TLR2, TLR3, TLR4 and TLR7 mRNA expression in pMDMs that were isolated from the peripheral blood mononuclear cells (PBMCs) of the Shimen-strain–infected pigs, attenuated vaccine pigs or CSFV–free pigs. Compared with the pMDMs of the CSFV–free group, the relative mRNA levels of porcine TLR2, TLR4 and TLR7 were significantly enhanced after 3 days of infection with the Shimen or C strain of CSFV (Fig. 2b). However, TLR3 was shown to be suppressed by infection with the Shimen strain and increased by infection with the C strain. The significance of the different results between the in vitro and in vivo CSFV infection is not currently understood. In the following studies, we focused on validating TLR2, TLR3, TLR4 and TLR7 gene expression in response to CSFV infection of pMDMs in vitro.

To further confirm the expression of TLR2, TLR3, TLR4 and TLR7 in CSFV–infected cells, we examined the protein levels by Western blotting in pMDMs infected with either the Shimen or C strain at several time points. As shown in Fig. 2(c), there was obvious upregulation of TLR2, TLR4 and TLR7 by the Shimen and C strains. Moreover, TLR3 showed almost an unaltered expression pattern in the presence of the C strain, but was significantly downregulated in Shimen-strain–inoculated pMDMs. Finally, using a flow cytometry assay on mock- or CSFV–treated cells, we found that the trends in TLR2, TLR3, TLR4 and TLR7 expression were correlated with the protein levels by Western blotting (Fig. 2d). Together, these results demonstrated that CSFV infection induced the expression of TLR2, TLR3, TLR4 and TLR7 at both the mRNA and protein levels; in particular, TLR3 expression was downregulated by the Shimen strain.

CSFV infection induced pro-inflammatory cytokines and the expression of IFNs in pMDMs

To ascertain whether CSFV infection triggered antiviral programmes and inflammatory responses that were downstream responses of TLR stimulation, we examined several cytokines and IFNs that were known to be induced by TLR activation by RT-PCR. As shown in Fig. 3(a), compared with the relative mRNA levels of the mock-inoculated pMDMs, Shimen- and C-strain–infected pMDMs expressed significantly higher levels of IL-1β, IL-8 and IFN-α, but IL-10, IL-12 p40 and TNF-α were found to be decreased by the C strain infection at the three time points. In contrast, IL-10 and IL-12 p40 expression increased remarkably when the cells were co-incubated with the Shimen strain, whereas the increase was not dramatic for TNF-α. Furthermore, IL-6 mRNA levels were significantly lower in C-strain–inoculated pMDMs than in the mock–infected cells; however, incubation with the Shimen strain resulted in a significant upregulation at 24 h post–infection (p.i.), but a downregulation at 48 and 72 h p.i.
TLR2 mRNA (relative to β-actin)

TLR4 mRNA (relative to β-actin)

TLR7 mRNA (relative to β-actin)

TLR3 mRNA (relative to β-actin)

Mock C 24 C 48 C 72 SM 24 SM 48 SM 72 (h p.i.)

Mock C strain SM strain

(a)

(b)
Fig. 2. TLR gene expression in pMDMs using different methods. (a) TLR (2, 3, 4 and 7) mRNA expression was analysed by RT-PCR in pMDMs that were mock-infected or infected with the Shimen (SM) or C strain of CSFV. Total RNA from the infected and uninfected cells was analysed at 24, 48 and 72 h post-infection (p.i.). (b) TLR mRNAs in the pMDMs of CSFV-infected pigs (Shimen strain), pigs inoculated with attenuated vaccine (C strain) and CSFV-free pigs (mock). The conditions of analysis were the same as in (a). (c) Western blotting of TLR (2, 3, 4 and 7) proteins in pMDMs at various time points after C or SM strain infection. β-Actin was analysed to verify equal loading. The relative levels of the targeted proteins are shown.
The relative expression of IFN-\(\beta\) in Shimen-strain-inoculated pMDMs was significantly decreased at the three time points, but was unaffected in the C-strain-infected cells. In addition, we found that the expression of IFN-\(\gamma\) did not differ markedly between the two groups.

To compare the effect of the expression of CSFV on TLR-dependent cytokine responses, and to investigate the specificity to TLR2, TLR3, TLR4 and TLR7, we used TLR-specific ligands (with reference to TLR activation controls) or the CSFV Shimen or C strains to treat pMDMs and measured the production of IFNs and pro-inflammatory cytokines (Fig. 3b). The results indicated that the Shimen strain of CSFV increased IL-1\(\beta\), IL-8 and IFN-\(\alpha\) secretion, but inhibited IL-6 and IFN-\(\beta\) production. Importantly, in pMDMs, stimulation with TLR2, TLR4 or TLR7 ligands showed the same pattern of IL-1\(\beta\) production as did the infection with the Shimen strain. The Shimen strain induced IFN-\(\alpha\) secretion with a trend similar to the TLR2, TLR3, TLR4 or TLR7 ligands, but suppressed the production of IFN-\(\beta\), as opposed to the TLR2, TLR3, TLR4 or TLR7 ligands, which showed significant activation of IFN-\(\alpha\) and IFN-\(\beta\).

**CSFV Shimen and C strains modulate TLR signalling pathways**

In view of the above-mentioned results, it was critical to analyse the mechanism of CSFV-mediated TLR signal transduction. For this purpose, we first examined the mRNAs of key proteins [i.e. MyD88 (myeloid differentiation factor-88), TRIF (TIR domain-containing adaptor inducing IFN-\(\beta\)), IFN regulatory factors (IRFs), NF\(\kappa\)B p65 and I\(\kappa\)B-\(\alpha\) (inhibitor of NF\(\kappa\)B)] in the TLR signal transduction pathways. Amongst them, in comparison with the control samples, the expression of MyD88 was significantly enhanced, but TRIF was suppressed in the Shimen- or C-strain-infected cells (Fig. 4a). Meanwhile, the transcription levels of IRF3 and IRF7 were unchanged after incubation with the C strain. Nevertheless, after Shimen strain infection, IRF3 expression was dramatically decreased whilst IRF7
expression was increased (Fig. 4a). Furthermore, NFκB p65 expression was not significantly influenced in either group; IkB-α gene expression was upregulated in pMDMs by infection with both the Shimen and C strains (Fig. 4a).

We then examined IRF3, IRF7, IkB-α, extracellular signal-regulated protein kinases ERK1/2, and the activation of p65 and ERK1/2 [a mitogen-activated protein kinase (MAPK) signalling pathway] in response to CSFV (Shimen and C strains).
strains) infection by Western blot. The results revealed that the production of IRF7 and ERK1/2 and the phosphorylation status of ERK1/2 were significantly activated by the Shimen strain in comparison with mock-infected cells; however, there was no apparent change in the levels of the phosphorylation status of NFκB p65 (Fig. 4b). Additionally, the protein levels of porcine IRF3 and IκB-α showed the same trends as the mRNA levels.

**CSFV Shimen strain modulates TLR3-mediated innate immune response**

To determine whether TLR3-induced innate responses were impaired in the presence of the Shimen strain, we first tested the expression of TLR3, TLR3 signalling intermediates (i.e. TRIF and IRF3) and downstream effector molecules (i.e. IL-6 and IFN-β) upon stimulating Shimen-strain-infected or mock-infected pMDMs with a high-purity TLR3 ligand [poly(I:C)] by RT-PCR. As observed in Fig. 5(a), the Shimen strain of CSFV also inhibited the expression of TLR3, TRIF, IRF3, IL-6 and IFN-β induced by treatment with the ligand.

We next examined the protein levels of TLR3, IRF3, IFN-β and IL-6, and the activation of NFκB p65 in response to stimulation by poly(I:C) in Shimen-strain-infected or mock-infected pMDMs. Western blot analyses revealed that the Shimen strain did not alter the phosphorylation status of NFκB p65, and exhibited a clear inhibition of the activation of TLR3 and IRF3 in response to stimulation with the TLR3 ligand poly(I:C) (Fig. 5b). Furthermore, ELISA showed that production of IL-6 and IFN-β was reduced in Shimen-strain-infected pMDMs by stimulation with poly(I:C) (Fig. 5c).
In our study, CSFV infection was found to induce differential gene expression of TLRs. Specifically, Shimen-strain-infected pMDMs showed an increased expression of TLR2, TLR4 and TLR7, and a decreased expression of TLR3; C-strain-infected pMDMs also exhibited a clear promotion of the expression of TLR2, TLR4 and TLR7, but had no effect on TLR3 expression. However, a contrary result was demonstrated in swine macrophages infected with the highly virulent CSFV strain Brescia (Borca et al., 2008). Therefore, we further examined the protein levels of TLRs using Western blotting and flow cytometry. Considering all of our results together, it was reasonable to conclude that both the Shimen and C strains showed the same trend in promoting the expression of TLR2, TLR4 and TLR7. However, the strongest finding of this study was the observation that TLR3 expression was downregulated by the Shimen strain. Thus, the consequences of TLR3 downregulation by the Shimen strain were investigated. The results once again clearly showed that TLR3-induced innate responses mediated by poly(I:C) were inhibited in the presence of the Shimen strain (Fig. 5).
MyD88 is the key adaptor protein of TLR-mediated signal transduction pathways, and shows significant activation of NFκB and MAPKs to induce the expression of pro-inflammatory cytokines (Saitoh et al., 2011). In contrast, TRIF is used by TLR3 and TLR4, and induces signalling pathways that lead to the activation of IRF3 and NFκB, and thus the consequent induction of type I IFN and pro-inflammatory cytokines (Kawai & Akira, 2011). In the present study, at the early stages (24, 48 and 72 h) of infection of pMDMs with the Shimen and C strains, MyD88 mRNA was found to be significantly upregulated; however, the expression of TRIF was downregulated, which implied that CSFV specifically inhibited the TLR/TRIF-dependent signalling pathway, whilst it promoted the TLR/MyD88-dependent signalling pathway.

NFκB is a key transcription factor in the induction of pro-inflammatory cytokines (Jeong et al., 2002). Activation of the MyD88-dependent pathway results in modulating NFκB-dependent transcription, followed by facilitating TNF-α and IL-6 induction (Kawai & Akira, 2010). Our results showed that both Shimen and C strain infection had no effect on the NFκB signalling pathway (probably related to the consequent induction of TNF-α and IL-6). This result was in agreement with a previous study that demonstrated that CSFV failed to activate the NFκB signalling pathway both in vitro and in vivo (Chen et al., 2012). The reason for this phenomenon may be a lack of IκB-α degradation (Fig. 4a, b).

In addition to NFκB activation, MAPKs also promote the secretion of pro-inflammatory cytokines. The activation of ERK1/2 in response to CSFV (Shimen and C strains) infection implies that, just as in the TLR/MyD88-dependent signalling pathway, the production of pro-inflammatory cytokines was probably due to the activation of MAPKs.

In the TLR signalling pathway, activation of IRF3 and IRF7 induces the expression of type I IFN (Honda et al., 2006). In accordance with a previous report (Bauhofer et al., 2007), the presence of the Shimen strain prevented IRF3 induction in this study. Consistent with those data, we can explain that after infection with the Shimen strain, the pMDMs inhibited the production of IFN-β protein. This finding is in accordance with the data in Fig. 2(c) showing TLR3 downregulation by the Shimen strain and
Fig. 5. CSFV Shimen strain modulates the TLR3-mediated innate immune response. Shimen-strain-infected or mock-infected pMDMs (18, 42 or 66 h) were stimulated for 6 h with 10 μg poly(I:C) ml⁻¹. (a) TLR3, TRIF, IRF3 and NFκB p65 mRNA expression levels in Shimen-strain-infected or mock-infected pMDMs either stimulated with poly(I:C) or untreated (control) were determined by RT-PCR. (b) Western blotting analysis of total cellular extracts for TLR3, IRF3 and the phosphorylation status of NFκB p65 in Shimen-strain-infected or mock-infected pMDMs stimulated with poly(I:C). β-Actin served as an internal control. The relative levels of the targeted proteins are shown 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 mock-inoculated or Shimen-infected pMDMs stimulated with poly(I:C) were measured by ELISA. Data are shown as the mean ± SD of three independent experiments. *P<0.05, **P<0.01 and ***P<0.001 calculated using Student’s t-test.

with data in Fig. 4(b) showing IRF3 downregulation. In contrast, the C strain produced no apparent change in the levels of TLR3, IRF3 and IFN-β.

Meanwhile, the Shimen strain induced much higher levels of IFN-α than the C strain during the infection of pMDMs. The IFN-α upregulation result was a complete contradiction of numerous reports demonstrating that CSFV-infected cells (Carrasco et al., 2004; Ruggli et al., 2003), except for plasmocytoid dendritic cells (Balmelli et al., 2005), do not produce any type I IFN due to the efficient elimination of IRF3 mediated by NPro (Bauhofer et al., 2007). It is noteworthy that IRF7 was essential for the production of IFN-α/β in distinct cell types upon viral infection (Honda et al., 2005) and IFN-α induction through the TLR/MyD88/IRF7-dependent signalling pathway (Kawai et al., 2004). This appeared consistent with the present report of the activation of TLR/MyD88/IRF7-dependent IFN-α production by Shimen strain infection in pMDMs. Similar observations have been reported in pigs infected with virulent strains of CSFV which induce high levels of IFN-α (not IFN-β) starting at the early phase (Borca et al., 2008; Ruggli et al., 2009; Summerfield et al., 2006).

A temporal pattern of accumulation or consumption of pro-inflammatory cytokines was observed upon infection of pMDMs with the Shimen or C strain. During virus infection, IL-1β production is induced by inflammasome signalling (Negash et al., 2013). The chemokine CXCL8 (IL-8) is involved in the activation and recruitment of immune cells into the infected tissues (Darwich et al., 2003). Our study showed that both CSFV strains could induce IL-1β and IL-8 expression, suggesting the occurrence of systemic inflammation following CSFV infection. The production of IL-10 is required for viral persistence (Brooks et al., 2006). We found that IL-10 was enhanced upon infection with the Shimen strain, but slightly decreased by C strain infection (Fig. 3a, b). This result showed that IL-10 may play an important role in establishing persistent infection by the Shimen strain.
IL-6 plays an important role as a cytokine in providing an efficient immune response during early events of CSFV infection (Sánchez-Cordón et al., 2005). Contrary to the observations showing increased levels of IL-6 expression in CSFV-infected macrophages in vitro (Borca et al., 2008) and in vivo (Sánchez-Cordón et al., 2005), our study demonstrated that the levels of IL-6 mRNA and protein were slightly upregulated at 24 h, but showed reductions at 48 and 72 h after infection by the CSFV Shimen strain. Based on this finding, we hypothesize that IL-6 may promote infection and viral replication during early stages, whereas the absence of IL-6 may result in virus persistence or delayed virus clearance.

The same interactions already described amongst these proteins involved in the TLR-related metabolic pathways in human/rodent systems have also been shown to similarly occur in swine. For instance, G- and U-rich ssRNA oligonucleotides derived from the foot-and-mouth disease virus (FMDV) genome have been reported to not only stimulate PBMCs to secrete cytokines, but also induce the activation of NFκB and the production of IFN-α via pTLR7 (Zhang et al., 2008). This finding reveals that immune activation of the ssRNA and oligonucleotides of FMDV can be mediated by TLR7, and that TLR7 or TLR8 has a relatively wide recognition of viral ssRNA in different species and virus. Furthermore, the pathway of TLR7-dependent induction of IFN-α in plasmacytoid dendritic cells by infected cells independent of virions has been observed for hepatitis C virus (Takahashi et al., 2010). Interestingly, this process is also very efficient for CSFV (Python et al., 2013). In this study, we found that stimulation with TLR2, TLR3, TLR4 or TLR7 ligands significantly increased the secretion of specific cytokines in pMDMs. This seems to be similar to the previous reports that these cytokines are related to TLR-dependent signalling pathways in human system (Abe et al., 2007; Chang et al., 2007). However, the roles of these TLRs in activating immune responses in swine are still poorly understood so that further investigation is necessary.

In conclusion, it was shown by several independent experimental approaches that TLRs were involved in CSFV Shimen and C strain infection of pMDMs. CSFV infection resulted in a significant induction of TLR2, TLR4 and TLR7, but TLR3 remained unchanged (C strain infection) or was decreased (Shimen virus infection) compared with control. Importantly, TLR3-mediated innate responses induced by poly(I:C) were inhibited in the Shimen-strain-infected pMDMs. We also revealed that CSFV Shimen and C strain infection of pMDMs led to the activation of MAPK signalling pathways, whilst it failed to activate NFκB. Furthermore, compared with C strain infection, the Shimen strain was very efficient at eliminating IRF3 expression, but enhancing IRF7 expression, thereby affecting the production of type I IFN responses.

**METHODS**

**Cells and viruses.** pMDMs were differentiated from the PBMCs of CSFV-negative healthy pigs (kindly provided by Professor Zhi-Zhong Jing, Lanzhou Veterinary Research Institute, China) using Ficoll-Paque (GE Healthcare) density (1.077 ± 0.001 g ml⁻¹) gradient centrifugation (Chang et al., 2007). Briefly, culture flasks were precoated with 2 % sterile, endotoxin-free gelatin for 2 h and then incubated with PBS (Gibco) for another 1 h before plating cells. After gradient centrifugation, PBMCs were seeded into the precoated flasks and allowed to adhere for 2 h in the incubator at 37 °C, 5 % CO₂. Adherent cells were removed from the flasks using a mixture of RPMI 1640 (Gibco) and 10 mM EDTA in a 1 : 1 dilution and plated at 5 × 10⁵ ml⁻¹ in fresh RPMI 1640 containing 20 % FBS for 8 days.

Two CSFV strains designated the Shimen strain and C strain were obtained from the Control Institute of Veterinary Bioproducts and Pharmaceuticals (Beijing, China) and propagated in CSFV-permissive swine testicular cells.

**Infection and immunity of animals.** CSFV-negative healthy pigs were divided into groups of three. The infection and immunity groups were intramuscularly injected with lml CSFV Shimen strain (10⁵ TCID₅₀ml⁻¹) or C strain vaccine (according to the manufacturer’s instructions). The control group was injected with PBS, pH 7.4. The cells were then incubated in RPMI 1640 containing 2 % FBS. At 0, 24, 48 and 72 h p.i., pMDMs from each group were isolated as described earlier. The conditions for the cellular analysis used here were also used in the setup of the in vitro infections of the pMDMs.

All animal procedures were approved and supervised by the Animal Care Commission of the College of Veterinary Medicine, Northwest A&F University.

**Viral infection.** The CSFV Shimen and C strains were added to the cell culture supernatants at m.o.i. 10 [the m.o.i. was determined and calculated using the same method described previously (Ning et al., 2014) using pMDMs grown to 70–80 % confluency]. After a 1 h incubation, the inoculum was removed, and the cells were rinsed with PBS, pH 7.4. The cells were then incubated in RPMI 1640 containing 2 % FBS. At 0, 24, 48 and 72 h p.i., the cell-free culture supernatants and cell lysates were harvested and stored at −80 °C until use.

**Western blot analysis.** Cell lysates were prepared from control and CSFV-stimulated (Shimen and C strains) samples by lysis in RIPA buffer with Halt Protease Inhibitor Cocktail and Halt Phosphatase Inhibitor Cocktail, and maintained by gentle shaking throughout the 15 min incubation on ice followed by centrifugation at 14000 g for 15 min. After quantification using a bicinchoninic acid assay, the supernatants were boiled with SDS sample loading buffer (five times), separated by 12 % SDS-PAGE and then transferred onto PVDF membranes (Millipore). The membranes were blocked with TBS/Tween 20 (TBST) containing 5 % skim milk at room temperature for 2 h and then incubated with primary antibody, i.e., anti-TLR2 antibody (aa 591–640), mouse mAb (clone 40C1285.6) to human TLR3 and anti-IRF3 (or IRF7) polyclonal antibody (C-termirus) (LSBio), mouse anti-TLR4 antibody (HTA125) (Abcam), goat anti-TLR7 antibody (N-20) (Santa Cruz), p44/42 MAPK (ERK1/2) (137E5) rabbit mAb and phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) antibody (Cell Signaling Technology), IκB-α (L26) and NFκB p65 (phospho-S536) polyclonal antibody (Bioworld) or mouse anti-β-actin (GeneTex), at 4°C overnight. After several washes with TBST, the membranes were then incubated with HRP-conjugated goat anti-rabbit (or mouse) IgG or rabbit anti-goat IgG Fc (LSBio) secondary antibody (1 : 5000) for 2 h at room temperature. Unbound antibodies were removed by TBST washes and the signal was detected using an image analyser system (Bio-Rad), with exposure times of 30 s to 4 min.
RT-PCR. RT-PCR was used to determine the relative mRNA expression of cytokines, chemokines, IFN, TLR genes and other key elements of the TLR signal transduction pathways as well as the number of virus copies. Total RNA was prepared from the pMDMs using an RNeasy Mini kit (Qiagen). First-strand cDNA was synthesized using a PrimeScript RT reagent kit with gDNA Eraser (TaKaRa). Each cDNA was estimated by a One Step SYBR PrimeScript PLUS RT-PCR kit (TaKaRa) according to the manufacturer’s protocol. To normalize the relative expression of each gene in cells to the total amount of RNA (except CSFV-specific detection), a housekeeping gene, β-actin, served as an internal control. pCMV-myc plasmid encoding CSFV NS2 protein was used to build a fluorescence quantitative standard curve for calculating the copy numbers of CSFV in different samples. Specific RT-PCR primers for each gene are described in Table 1.

Flow cytometry analysis. pMDMs were infected with the C or Shimen strain for 48 h and then detached from plates using trypsin (HyClone). Dissociated cell aggregates were then centrifuged and cells resuspended in an appropriate volume of Flow Cytometry Staining Buffer so that the final cell concentration was $2 \times 10^7$ ml$^{-1}$.

For staining cell surface antigens, we combined the recommended quantity of primary antibody [i.e. anti-TLR2 antibody (aa 591–640) (LSBio) and anti-TLR4 antibody (HTA125) (Abcam)] in each cell suspension and incubated for at least 60 min in the dark on ice. Then, cells were washed two times with Flow Cytometry Staining Buffer, followed by centrifugation at 400 g at 4°C for 5 min. Next, we added the appropriate FITC-conjugated secondary antibody to the cells and incubated for 30 min in the dark on ice.

For staining intracellular antigens, cells were fixed by IC Fixation Buffer whilst vortexing the tube and incubated in the dark at room temperature for 20 min. Next, we added the recommended amount of each primary antibody [i.e. anti-TLR3 antibody (clone 40C1285.6) (LSBio) and anti-TLR7 antibody (N-20) (Santa Cruz)] and incubated at room temperature for 1 h in the dark, followed by the appropriate FITC-conjugated secondary antibody incubated at room temperature for 1 h in the dark. All washes after incubation with IC Fixation Buffer or antibody were with 2 ml 1× Permeabilization Buffer, followed by centrifugation at 400 g for 5 min, and then the supernatant was discarded.

Cell fluorescence was analysed using an EPICS XL (Beckman, USA) flow cytometer and EXPO32 analysis software (Beckman). FITC-conjugated IgG was used as an isotype control.

ELISA. Cells were seeded onto 24-well plates at a concentration of $4 \times 10^5$ cells per well and stimulated with TLR-specific ligands.

Table 1. Host genes analysed and primer sets used in this study

<table>
<thead>
<tr>
<th>Genes</th>
<th>Forward primer (5’→3’)</th>
<th>Reverse primer (5’→3’)</th>
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<td>CATGTCCTGTCATGACTATCC</td>
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<td>Cytokines and chemokine</td>
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<tr>
<td>IL-1β</td>
<td>CACGCCCTCTCAAGAGAACA</td>
<td>GGCAGCAACCATGACCAACT</td>
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<td>IL-6</td>
<td>GAGCCCAACAGAGAAGAGA</td>
<td>GCAGTACACACCATGACAGAAGA</td>
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<td>IL-8</td>
<td>TTCTATGCGATGCTATGAAAT</td>
<td>CTCGGACACCTTATGACCAAC</td>
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<td>IL-10</td>
<td>CATGCGGCGGACTTGTTG</td>
<td>ACAGGGCAAGAAATGTGAC</td>
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<tr>
<td>IL-12 p40</td>
<td>CAGCGCAAGCTCCTACGA</td>
<td>CTCGAGACACCTCTCTCTG</td>
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<tr>
<td>TGF-β</td>
<td>CCGATCGCGCTCTCCTACCA</td>
<td>CTGCGACATGCAACAGAATTC</td>
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<tr>
<td>Key elements in TLR signal transduction pathways</td>
<td></td>
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<tr>
<td>MyD88</td>
<td>GCCGTCGATGAGTGGTGTGTTG</td>
<td>TGGTGACAGGTTGTTGTTGATG</td>
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<tr>
<td>TRIF</td>
<td>TGGGACATCTTATAGGAGAAT</td>
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<tr>
<td>IRE3</td>
<td>CATACACTTGCTTGTGTTGATGAC</td>
<td>TCTGCTCTGCTTGTGTTGATG</td>
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<tr>
<td>IRE7</td>
<td>TGGATGCGCTTGATGAGA</td>
<td>AGGGACACGGGAGATG</td>
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<td>NFκB p65</td>
<td>GGGACTACGACCTTGAAATGCT</td>
<td>GGGGAGGTTGTTGCAAGAT</td>
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<td>IκB-α</td>
<td>GAAGGTGAAAGTCTCTG</td>
<td>TCGTACATATTAGTGCCCTG</td>
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</table>
[PGN-SA (InvivoGen), poly(IC), LPS (Sigma) or R-837 (InvivoGen), which are ligands for TLR2, TLR3, TLR4 and TLR7, respectively] or with CSEV Shimen or C strain. Cell culture supernatants were collected at different time points and analysed using a Duoset ELISA Development System for porcine IL-1β, IL-6, IL-8 and IL-12 p40 (R&D Systems). The secreted protein levels of IFN-α and IFN-β were determined with IFN-α antibody (G16) and IFN-β antibody (LSBio), 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).

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**REFERENCES**


