Porcine epidemic diarrhea virus infection induces NF-κB activation through the TLR2, TLR3 and TLR9 pathways in porcine intestinal epithelial cells

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Porcine epidemic diarrhea virus (PEDV) is a coronavirus that induces persistent diarrhoea in swine, resulting in severe economic losses in swine-producing countries. Insights into the interplay between PEDV infection and the innate immune system are necessary for understanding the associated mechanism of pathogenesis. The transcription factor NF-κB plays an important role in regulating host immune responses. Here, we elucidated for the first time to our knowledge the potential mechanism of PEDV-mediated NF-κB activation in porcine small intestinal epithelial cells (IECs). During PEDV infection, NF-κB p65 was found to translocate from the cytoplasm to the nucleus, and PEDV-dependent NF-κB activity was associated with viral dose and active replication. Using small interfering RNAs to screen different mRNA components of the Toll-like receptor (TLR) or RIG-I-like receptor signalling pathways, we demonstrated that TLR2, TLR3 and TLR9 contribute to NF-κB activation in response to PEDV infection, but not RIG-I. By screening PEDV structural proteins for their ability to induce NF-κB activities, we found that PEDV nucleocapsid protein (N) could activate NF-κB and that the central region of N was essential for NF-κB activation. Furthermore, TLR2 was involved in PEDV N-induced NF-κB activation in IECs. Collectively, these findings provide new avenues of investigation into the molecular mechanisms of NF-κB activation induced by PEDV infection.

INTRODUCTION

Infection with porcine epidemic diarrhea virus (PEDV) is associated with high morbidity in animals of all ages and results in high mortality rates (up to 100%) in suckling piglets, with clinical symptoms such as vomiting, acute watery diarrhoea and dehydration (Debouck & Pensaert, 1980). Following the initial discovery of PEDV in 1972, it did not emerge in North America until 2013, after which it rapidly spread to many countries (Ojkic et al., 2015; Vlasova et al., 2014). Starting in late 2013, outbreaks of porcine epidemic diarrhea (PED) continued to increase and cause devastating economic effects among swine-producing Asian countries (Lin et al., 2014; Park et al., 2014); these outbreaks have received considerable attention in recent years.

PEDV, a member of the order Nidovirales and family Coronaviridae, is an enveloped virus containing a single-stranded, positive-sense RNA genome of 28 kb with a 5’ cap and a 3’-polyadenylated tail (Eggerink et al., 1988; Kocherhans et al., 2001). PEDV encodes four structural proteins, namely the spike protein (S; 150–220 kDa), membrane protein (M; 20–30 kDa), envelope protein (E; 7 kDa) and nucleocapsid protein (N; 58 kDa), which are all located in the 3’ third of the PEDV genome.

During viral infection and replication, innate host immune responses serve as the first line of defence. NF-κB is a transcription factor that plays an important role in the regulation of immune responses, involved in modulating the expression of multiple pro-inflammatory cytokines, chemokines, adhesion molecules and inducible enzymes, as well as cell survival/proliferation (Moynagh, 2005). However, overactivation of NF-κB can result in inflammatory and autoimmune diseases; therefore, activation of this transcription factor is thought to be a hallmark of most infections (DeDiego et al., 2014; Yamamoto & Gaynor, 2001). Innate immune responses are activated by host pattern-recognition receptors (PRRs). Retinoic acid-inducible gene-1 (RIG-I)-like receptors (RLRs) and Toll-like receptors (TLRs) serve as two major PRRs that can detect viral pathogen-associated molecular patterns (PAMPs). After
recognizing the PAMPs, these receptors interact with their corresponding adaptor molecules to elicit downstream signalling events, resulting in the activation of NF-κB and IFN-regulatory factors that induce several types of antiviral cytokines (Thompson & Locarnini, 2007). NF-κB belongs to the Rel protein family, which contains NF-κB p50 (p105/NF-κB1), NF-κB p52 (p100/NF-κB2), p65 (RelA), RelB and c-Rel. In resting cells, NF-κB exists in the cytoplasm as an inactive homodimer or heterodimer bound to the inhibitory protein IκB. NF-κB is released from the NF-κB/IκB complex by proteasomal degradation of IκB in response to exogenous stimuli (e.g. pro-inflammatory factors and PAMPs), leading to the nuclear translocation of NF-κB (May & Ghosh, 1998).

Most studies of PED have focused mainly on pathogen isolation and identification, genome and structural protein analysis, diagnosis and vaccine development (Song & Park, 2012); however, the detailed molecular mechanism of viral pathogenesis remains unknown. The outcomes of viral infection are primarily determined by the interplay between the virus and the innate immune system (Frieman et al., 2008). Therefore, insights into how the virus interacts with the host innate immune system are necessary to understand the molecular mechanisms of viral pathogenesis. Results from previous reports suggest that PEDV does not induce type I IFN production and that IFN activity may be antagonized by the PEDV-encoded non-structural protein papain-like protease 2 (PLP2) and the structural N protein (Ding et al., 2014; Xing et al., 2013). In addition, evidence has shown that stable expression of the PEDV N and E proteins in porcine intestinal epithelial cells (IECs) can activate NF-κB and upregulate IL-8 expression (Xu et al., 2013a, b). Ding et al. (2014) reported that transient expression of the PEDV N protein could inhibit NF-κB activation in HEK-293T cells. However, the precise mechanisms regulating NF-κB activity during PEDV infection remain unclear.

PEDV, transmissible gastroenteritis virus (TGEV) and porcine rotavirus are major swine pathogens that cause viral gastroenteritis, and the target of these viruses is the intestinal epithelium (Liu et al., 2010; Zhao et al., 2014). Therefore, IECs are optimal for studying the pathogenic mechanisms of these viruses, owing to their strong similarities with primary intestinal epithelial cells. In this study, we investigated PEDV-induced NF-κB activation and also observed the potential molecular mechanisms of PEDV-mediated NF-κB activation in IECs.

RESULTS

IECs are permissive for PEDV infection

Immunofluorescence assay (IFA) experiments were performed to determine whether PEDV strain CV777 could infect IECs. IECs were incubated with PEDV at an m.o.i. of 0.1, 0.01 or 1. At 48 h post-infection (p.i.), we observed significantly higher fluorescence in IECs infected with PEDV at an m.o.i. of 1 compared with that observed in cells infected at an m.o.i. of 0.1 or 0.01 (Fig. 1a). IFA intensities were nearly undetectable in IECs infected with PEDV at an m.o.i. of 0.01. Next, we observed the replication of PEDV at different time points p.i. (12, 24, 36 and 48 h) in IECs infected with PEDV at an m.o.i. of 1 (Fig. 1b). The results showed a time-dependent increase in fluorescence intensity beginning at 24 h p.i. (Fig. 1b). In conclusion, the IECs were permissive for PEDV infection.

PEDV infection activates NF-κB induction

The intestinal epithelial cells (IECs) not only have a physical barrier, but can also trigger innate immune responses

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**Fig. 1.** Infection of PEDV in IECs. (a) IECs were infected with PEDV at an m.o.i. of 0.01, 0.1 or 1 for 48 h, and PEDV infection was analysed by IFA. (b) IECs were infected with PEDV at an m.o.i. of 1. Cells were fixed at different time points p.i. (12, 24, 36 and 48 h), and PEDV replication was detected in IFA assays.
against foreign agents, such as bacteria and viruses (Schierack et al., 2006). Luciferase reporter assays were performed to determine whether PEDV infection can activate the NF-κB signalling pathway in IECs. IECs were cotransfected with the pNF-κB-Luc and the internal-control phRL-TK plasmids, and the cells were then infected with PEDV (m.o.i. 0.01, 0.1 or 1). At 24 h p.i., the cells were treated with TNF-α (10 ng) or re-transfected with poly(I:C) (1 μg) for 12 h. TNF-α and poly(I:C) are known as positive stimuli of NF-κB. As shown in Fig. 2(a), positive stimulation with TNF-α or poly(I:C) induced high expression of the NF-κB-regulated luciferase, and this activity increased in PEDV-infected IECs in a dose-dependent manner compared with that observed in mock-infected IECs. Compared with mock-infected cells, pNF-κB-Luc induction increased by 1.3-, 1.5- and 1.9-fold in IECs infected with PEDV at an m.o.i. of 0.01, 0.1 or 1, respectively, by 2.3-fold in poly(I:C)-transfected cells, and by 2.6-fold in TNF-α-treated cells.

Because nuclear translocation is an essential step for NF-κB activity, we next tested whether PEDV infection led to translocation of the NF-κB p65 subunit from the cytoplasm to the nucleus. Western blot analysis indicated that in IECs infected with PEDV (m.o.i. 1), nuclear accumulation of the p65 protein was increased by PEDV infection at 12 h p.i., which was significantly enhanced at 36 and 48 h p.i. (Fig. 2b). We next detected PEDV-induced NF-κB nuclear translocation by confocal microscopy. IECs were transfected with p65-GFP expression plasmids, followed by PEDV infection at an m.o.i. of 1. Poly(I:C)-transfected cells were used as a positive control. p65-GFP showed a punctate cytoplasmic distribution in mock-infected IECs [Fig. 2c(i)], and nuclear localization of p65-GFP was observed when the IECs were stimulated with PEDV infection or poly(I:C) transfection (Fig. 2c(ii, iii)). Taken together, our data clearly implied that PEDV infection could activate the nuclear translocation of NF-κB.

**PEDV-induced NF-κB activation is closely related to viral replication**

Results from previous reports have shown that NF-κB activation by some viruses depends mainly on efficient viral replication (Lee & Kleiboeker, 2005; Liu et al., 2008). To identify whether PEDV also exhibits this characteristic, the pNF-κB-Luc and phRL-TK plasmids were co-transfected into IECs, and the cells were then infected with active PEDV or UV-inactivated PEDV at an m.o.i. of 1. As shown in Fig. 3, NF-κB luciferase activities tended to increase during the progression of PEDV infection from 24 to 48 h p.i., and this increase was significantly different compared with mock-infected IECs (P < 0.01). In contrast, UV-inactivated PEDV did not induce NF-κB-regulated gene expression following its addition to the IECs at the same times. These results suggested that NF-κB activation was primarily due to active PEDV replication.

**Fig. 2.** PEDV induces NF-κB activation in IECs. (a) IECs were co-transfected with pNF-κB-Luc and phRL-TK, then infected with PEDV at an m.o.i. of 0.01, 0.1 or 1 for 24 h. Cells were treated with either poly(I:C) or TNF-α for 12 h, harvested and analysed in dual-luciferase assays. Firefly luciferase activity was normalized by Renilla luciferase activity. Data are displayed as relative fold changes in luciferase activities, compared with the activity observed in mock-infected cells. All data are expressed as the mean ± SD (**P < 0.01) relative to mock-infection controls. (b) IECs were infected with PEDV at an m.o.i. of 1. At 6, 12, 24, 36 and 48 h p.i., nuclear extracts were prepared and subjected to Western blot analysis. (c) IECs were transfected with the p65-GFP fusion protein vector, followed by (i) mock or (ii) PEDV infection for 24 h. (iii) Selected cells were retransfected with poly(I:C) as positive control. p65 nuclear translocation was visualized by confocal microscopy. p65-GFP was expressed in IECs (green), cells were stained with an anti-PEDV antibody (red) and nuclei were stained by DAPI (blue). Initial magnification, × 20. All experiments were performed in triplicate.
Silencing TRIF and MyD88 expression, but not RIG-1 expression, inhibits PEDV-induced NF-κB activation

RIG-I is a member of the RLR family, members of which are intracellular sensors of double-stranded RNA, and TRIF and MyD88 are two primary adaptor molecules of TLRs. To identify associations of the RLR and TLR signalling pathways with PEDV-induced NF-κB activation in IECs, we silenced the expression of RIG-I, TRIF and MyD88 with small interfering RNAs (siRNAs). Real-time reverse-transcription (RT)-PCR was employed to evaluate the interference effects of the siRNAs in IECs. siRNAs targeting RIG-I, TRIF and MyD88 significantly suppressed the mRNA expression of the corresponding targets, compared with a negative-control (NC) siRNA (Fig. 4a). After validating the successful downregulation of RIG-I, TRIF and MyD88 expression with siRNAs, the IECs were co-transfected with pNF-κB-Luc, phRL-TK and siRNAs for 12 h, after which the cells were infected with PEDV (m.o.i. 1) for an additional 36 h. As shown in Fig. 4(b), siTRIF and siMyD88 significantly decreased PEDV-induced NF-κB luciferase activity, while no significant change was detected in siRIG-I transfectants compared with NC siRNA transfectants. We then determined by Western blotting analysis whether the p65 concentration in nuclear extracts was decreased in the siRNA-transfectants after PEDV infection. As Fig. 4(c) shows, PEDV-induced p65 protein expression in nuclear extracts was significantly inhibited by TRIF- and MyD88-specific siRNA, in comparison with NC; however, it was not affected by RIG-1-specific...

Fig. 3. PEDV-induced activation of NF-κB depends on viral replication. IECs were co-transfected with the pNF-κB-Luc and phRL-TK plasmids, then infected with PEDV or UV-inactivated PEDV at an m.o.i. of 1. Cells were harvested at 12, 24, 36 or 48 h p.i., and the lysates were analysed in dual-luciferase assays. Firefly luciferase activity was normalized by Renilla luciferase activity. Data are displayed as relative fold changes in luciferase activities, compared with the activity observed in mock-infected cells. All data are expressed as the mean ± SD of three independent experiments. **P<0.01, relative to mock-infection controls.

Fig. 4. PEDV-induced activation of NF-κB depends on TRIF and MyD88, but not RIG-1. (a) Silencing efficiency of siRNAs targeting TRIF, MyD88 or RIG-I. IECs were transfected with the siRNAs indicated for 24 h. Total RNA was extracted and target gene or β-actin mRNA expression was analysed by real-time RT-PCR. Relative amplification of the target gene expression levels was normalized to β-actin expression. All data are expressed as the mean ± SD of three independent experiments. **P<0.01, relative to NC. (b) IECs were co-transfected with the pNF-κB-Luc and phRL-TK plasmids, as well as siTRIF, siMyD88, siRIG-I or NC siRNA for 12 h. Cells were infected or mock infected with PEDV for an additional 36 h. Cells were then harvested and analysed in luciferase reporter assays. Firefly luciferase activity was normalized by Renilla luciferase activity. Data are displayed as relative fold changes in luciferase activities, compared with the activity observed in NC siRNA-transfected, mock-infected controls. All data are expressed as the mean ± SD of three independent experiments. **P<0.01, relative to NC siRNA-transfected, PEDV-infected controls. (c) IECs were transfected with siTRIF, siMyD88, siRIG-I or NC siRNA for 12 h. Cells were infected or mock infected with PEDV for an additional 36 h. Cells were then harvested and analysed in luciferase reporter assays. Firefly luciferase activity was normalized by Renilla luciferase activity. Data are displayed as relative fold changes in luciferase activities, compared with the activity observed in NC siRNA-transfected, mock-infected controls. All data are expressed as the mean ± SD of three independent experiments. **P<0.01, relative to NC siRNA-transfected, PEDV-infected controls. (c) IECs were transfected with siTRIF, siMyD88, siRIG-I or NC siRNA, as indicated. Twelve hours later, cells were mock infected or infected with PEDV for an additional 36 h. p65 expression in nuclear extracts was detected by Western blot analysis. These experiments were performed in duplicate.
siRNA. These results suggested that the TLR signalling pathway is involved in PEDV-induced NF-κB activation.

**Silencing of TLR2, TLR3 or TLR9 inhibited PEDV-induced NF-κB activation**

Different TLRs, such as TLR1, TLR2, TLR3, TLR4, TLR6, TLR8, TLR9 and TLR10, are expressed in porcine small IECs (Arce et al., 2010). TLR3, TLR8 and TLR9 have been reported to recognize different types of viral nucleic acid, and TLR2 and TLR4 can recognize viral proteins (Thompson & Locarnini, 2007). To identify the role of individual TLR family members in PEDV-induced NF-κB activation in IECs, specific siRNA molecules targeting TLR2, TLR3, TLR4, TLR8 and TLR9 were synthesized and tested. The silencing efficiency of siRNA against these receptors exceeded 55% (Fig. 5a). Next, IECs were co-transfected with the pNF-κB-Luc and phRL-TK plasmids, and either a TLR-specific siRNA or the NC siRNA. Twelve hours later, the cells were mock infected or infected with PEDV at an m.o.i. of 1 for an additional 36 h. Knockdown of TLR2, TLR3 or TLR9 expression dramatically blocked PEDV-induced NF-κB activation, while pNF-κB-Luc activity was not inhibited in cells transfected with siRNAs targeting TLR4 or TLR8, compared with NC siRNA transfectants (Fig. 5b). We also investigated changes in nuclear p65 protein accumulation in siRNA-transfected cells following PEDV infection. Compared with the NC siRNA, TLR2-, TLR3- and TLR9-specific siRNAs inhibited PEDV-induced nuclear p65 protein expression, while TLR4- and TLR8-specific siRNAs did not (Fig. 5c). These data indicated that TLR2, TLR3 and TLR9 could activate NF-κB in response to PEDV infection.

**PEDV-encoded nucleocapsid protein activates NF-κB**

PEDV encodes four structural proteins. To investigate whether these proteins are involved in NF-κB activation, IECs were co-transfected with pNF-κB-Luc, phRL-TK and plasmids expressing the PEDV S, M, N or E protein. As shown in Fig. 6(a), overexpression of S, M or E did not stimulate NF-κB-regulated gene expression, compared with that observed following TNF-α treatment or poly(I:C) transfection, but this activity was significantly enhanced in IECs overexpressing PEDV N. In addition, N effectively increased NF-κB-regulated luciferase activity in a dose-dependent manner (Fig. 6b). To confirm that the PEDV N protein could induce NF-κB activation, we studied its effect on nuclear translocation of the NF-κB subunit p65 by confocal microscopy. As shown in Fig. 6(c), in control-vector-transfected IECs, p65-GFP was mainly expressed in the cytoplasm [Fig. 6c(i)]. However, upon PEDV N protein overexpression in transfected IECs, the punctate distribution of the p65-GFP was detected in the nucleus [Fig. 6c(ii)]. Taken together, these results demonstrated the capacity of the PEDV N protein to stimulate NF-κB activation.

**Fig. 5.** Silencing of TLR2, TLR3 or TLR9 expression decreases PEDV-induced activation of NF-κB. Experiments were performed similarly to those described in the legend of Fig. 4, except that siRNAs targeting TLR2, TLR3, TLR4, TLR8 or TLR9 were used. (a) The silencing efficiency of the siRNAs indicated was analysed by real-time RT-PCR. (b) The effects of siTLRs on PEDV-induced NF-κB activation were detected in luciferase reporter assays. (c) Cells were transfected with siTLRs prior to PEDV infection as indicated, and nuclear p65 expression was assayed by Western blot analysis.

The PEDV N protein contains a nucleolar localization signal (NoLS) sequence located at aa 71–90, and two nuclear export signal (NES) sequences at aa 221–236 (NES-1) and 325–364 (NES-2) (Shi et al., 2014). We analysed the structure of the PEDV N protein using Lasergene DNASTAR Protean 9.0 software, finding that the immunodominant region of the PEDV N protein is mainly localized to aa 136–289 and that this region has high hydrophilicity and flexibility. To investigate which domain of the PEDV N protein is necessary for NF-κB activation, we first cloned seven deletion mutants of the N protein into the eukaryotic expression plasmid pcDNA3.1,
Fig. 6. The PEDV-encoded nucleocapsid protein activates NF-κB signalling. (a) IECs were co-transfected with pNF-κB-Luc, phRL-TK and PEDV structural protein expression plasmids (S, M, N or E) or a negative-control vector for 24 h. Two groups of cells transfected with the vector control were re-transfected with poly(I:C) or treated with TNF-α for another 12 h. The cells were lysed and analysed in dual-luciferase assays. Firefly luciferase activity was normalized by Renilla luciferase activity. Data are displayed as relative fold changes in luciferase activity, compared with the activity observed in vector-control-transfected cells. All data are expressed as the mean ± SD of three independent experiments. **P<0.01, compared with control-vector transfectants. (b) IECs were co-transfected with pNF-κB-Luc, phRL-TK and PEDV N expression plasmids (0, 0.1, 0.2, 0.4 or 0.6 μg) for 36 h. Cells were then analysed in dual-luciferase assays. Data are displayed as relative fold changes in luciferase activity, compared with that observed in control-vector transfectants. All data are expressed as the mean ± SD of three independent experiments. a, <0.05 compared with vector-control cells; b, <0.05 compared with 0.1 μg N-protein vector transfected cells; c, <0.05 compared with 0.2 μg N-protein vector transfected cells. (c) IECs were co-transfected with the p65-GFP fusion protein vector and (i) an empty vector or (ii) the PEDV N expression vector for 24 h. NF-κB p65 (green), PEDV N (red) and the nuclei (blue) were visualized by confocal microscopy. Initial magnification, × 20. (d) Schematic representation of the PEDV N-protein serial-deletion mutant constructs. (e) IECs were co-transfected with pNF-κB-Luc, phRL-TK and PEDV N deletion-mutant expression plasmids. Twenty-four hours later, selected cells were stimulated with poly(I:C) or TNF-α for 12 h, and the cells were then harvested for NF-κB luciferase activity assays. Data are displayed as relative fold changes in luciferase activities, compared with that observed in control-vector transfectants. All data are expressed as the mean ± SD of three independent experiments. *P<0.05; **P<0.01, compared with N.
which were designated N70 (aa 1–70), N135 (aa 1–135), N289 (aa 1–289), ΔNAC (aa 136–289), C136 (aa 136–442), C290 (aa 290–442) and C365 (aa 365–442), respectively (Fig. 6d). The relationships between these mutants and NF-κB activation were analysed in luciferase reporter assays. As shown in Fig. 6(e), ΔNAC and C136, which contain NES-1, NES-2 and the immunodominant region (but without NoLS, C-terminal and N-terminal), enhanced NF-κB luciferase activity comparably to the full-length PEDV N protein. N289, containing NES-1 and the immunodominant region, also augmented the pNF-κB-Luc activity, but this augmentation was significantly decreased compared with that of ΔNAC and C136, suggesting that NES-1 (aa 221–236) may be essential for NF-κB activation. In addition, NF-κB luciferase activity was sharply decreased with the other N-protein deletion mutants lacking this domain, compared with the full-length PEDV N protein, suggesting that the central region may be critical for NF-κB activation by the full-length N protein.

**TLR2 mediates PEDV N-protein-induced NF-κB activation**

Previous experiments showed that TLR2 was involved in PEDV-induced NF-κB activation. Subsequently, it was investigated whether PEDV N-protein-induced NF-κB activation depends on TLR2 signalling. As shown in Fig. 7(a), pre-treatment of cells with siTLR2 impaired NF-κB activation by PEDV N protein in comparison with IECs transfected with NC siRNA. We next tested the accumulation of nuclear p65 in TLR2-silenced IECs in response to the PEDV N protein. As shown in Fig. 7(b), when overexpressing the PEDV N protein in IECs, the level of nuclear p65 was inhibited by siTLR2, compared with NC siRNA. These results suggested that TLR2 may be responsible for PEDV N-protein-induced NF-κB activation.

**DISCUSSION**

Vero E6 cells are the only continuous cell line that is highly permissive for PEDV infection in *vitro* (Hofmann & Wyler, 1988), and they are often used for viral propagation and protein analysis. Nonetheless, the *in vivo* target cells of PEDV are IECs. Infection of intestinal epithelium causes cytoplasmic vacuole formation and subsequent desquamation, resulting in digestive disorders. Data from previous studies have shown that differences occur in the immune functions of porcine epithelial cells and their interactions with other immune cell populations, compared with those from other species (Schierack *et al.*, 2006). Epithelial cells play a pivotal role in coordinating host immune responses via production of several essential signalling molecules (such as cytokines and chemokines, as well as cell surface molecules), the expression of which is also regulated by NF-κB (Moynagh, 2005; Pitman & Blumberg, 2000).

Therefore, in this study, to identify the mechanism of PEDV-induced NF-κB activation more accurately, IECs were used as host cells. We found that PEDV infection of IECs led to the nuclear translocation of NF-κB (Fig. 2b, c). The activation of NF-κB by PEDV was dependent on the viral dose and active replication, as determined using a luciferase reporter assay (Figs. 2a and 3).

Viruses can escape host immune responses by regulating NF-κB activation. Previous data have indicated that nidoviruses, including severe acute respiratory syndrome coronavirus (SARS-CoV) and Middle East respiratory syndrome coronavirus (MERS-CoV), have developed strategies to evade innate immunity by interfering with NF-κB signalling pathways (DeDiego *et al.*, 2014; Matthews *et al.*, 2014). In contrast, TGEV, murine herpesvirus, and
porcine reproductive and respiratory syndrome virus (PRRSV) can replicate more efficiently by activating NF-κB signalling (Eleouet et al., 1998; Haas et al., 2014; Lee & Kleiboeker, 2005). Although the NF-κB pathway is known to play an important role in immune responses, no previous evidence has suggested that PEDV infection regulates this pathway. However, data present in this study offer novel insights into the mechanism by which PEDV infection can initiate NF-κB activation.

NF-κB is one branch of the signalling pathway downstream of TLRs and RLRs. Therefore, some viruses, such as Epstein–Barr virus (Samanta et al., 2006), the human immunodeficiency virus (HIV) (Berg et al., 2012) and bovine herpesvirus 1 (da Silva & Jones, 2012), can induce NF-κB activation by the RLR-mediated pathway. In contrast, other viruses such as herpes simplex virus type 1 (Liu et al., 2008; Takeda et al., 2011), leukaemia virus (Abujamra et al., 2006) and PRRSV (Song et al., 2013) can induce NF-κB activation by the TLR-mediated pathway. By siRNA screening, our studies showed that RIG-I knockdown did not impede PEDV-initiated NF-κB activation; however, silencing the TLR adaptor molecules TRIF and MyD88 clearly impaired PEDV-initiated NF-κB activity (Fig. 4b, c). Further experimental data suggested that silencing TLR2, TLR3 or TLR9 expression inhibited PEDV-induced NF-κB activity (Fig. 5b, c). We also assessed the combined effects of siTLR2, siTLR3 and siTLR9 on PEDV-induced NF-κB activation in IECs. Unexpectedly, we found that the combined effects of these three siRNAs were similar to the effects observed when the siRNAs were transfected individually (data not shown). In conclusion, these data imply that NF-κB activation by PEDV infection mainly depends on the TLR signalling pathway.

It is clear that viral gene products can positively modulate NF-κB activity. Examples of such viral proteins include the PRRSV structural protein N and the non-structural protein 2 (NSP2) (Fang et al., 2012; Fu et al., 2012; Luo et al., 2008), herpes simplex virus glycoproteins gH/gL and gB (Leoni et al., 2012), Epstein–Barr virus latent membrane protein 1 (Smirnova et al., 2011), HIV envelope glycoprotein gp120 (Bren et al., 2009), SARS-CoV proteins S and N (Dosch et al., 2009; Liao et al., 2005), and hepatitis C virus protein NS2 (Oem et al., 2008). Virion envelope proteins could initiate NF-κB activation by binding to cellular surface receptors. Our observations showed that UV-inactivated PEDV failed to active NF-κB. In addition, while screening PEDV structural proteins for their ability to induce NF-κB activation, we found that the S, M and E proteins, located in the virion envelope, could not activate NF-κB. It is possible that the interaction between these virion envelope proteins and cellular surface receptors is not required for PEDV-inducing NF-κB activation. Surprisingly, the TLR2 cell surface receptor was involved in NF-κB activation following PEDV infection, suggesting that the phenomenon of enhanced NF-κB activation occurs via other mechanisms. The PEDV N protein, which binds virion RNA to form a helical capsid, significantly increased NF-κB activity, and TLR2 signalling was involved in PEDV N-protein-induced NF-κB activation (Figs. 6 and 7). These results suggested that PEDV N protein might activate NF-κB indirectly. As mentioned above for other viruses, non-structural PEDV may also be involved in regulating NF-κB activation, although further studies are needed to clarify this.

In summary, the aim of the present study was to shed insight into the potential mechanism whereby PEDV infection induces NF-κB activation in IECs. Our results suggest that when IECs are infected with PEDV, TLR2, TLR3 and TLR9 may serve to activate the NF-κB signalling pathway. Our experimental data further demonstrate that the PEDV N protein can activate NF-κB in IECs and that PEDV N-protein-induced NF-κB activation depends on TLR2 signalling. However, it is well known that viruses regulate NF-κB activation through multiple signalling pathways. Here, the mechanisms of PEDV-induced activation of NF-κB were only addressed by the RLR and TLR signalling pathways in vitro; therefore, additional studies are needed in the future. Understanding the PEDV-dependent effects on NF-κB activation should provide key information regarding the molecular pathogenesis of PEDV infection.

**METHODS**

**Cells and virus.** The African green monkey kidney cells (Vero E6) and PEDV strain CV777 were maintained in our laboratory, and porcine small intestinal epithelial cells (IECs) were isolated from the ileum of a newborn piglet (Xu et al., 2013a, b). Vero E6 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) with 8% FBS and were used to propagate PEDV, as described by Hofmann & Wyler (1988). IECs were cultured in Dulbecco’s modified Eagle’s F12 Ham medium (DMEM-F12) supplemented with 5% FBS.

**Reagents and antibodies.** TNF-α was purchased from PeproTech and reconstituted in water to a concentration of 0.1 mg ml⁻¹. Poly(I:C) was purchased from Invitrogen and dissolved in water to a concentration of 10 mg ml⁻¹. The Dual-Luciferase Reporter Assay System was purchased from Promega. Anti-p65 rabbit polyclonal antibodies and horseradish peroxidase (HRP)-conjugated goat anti-rabbit or goat anti-mouse IgGs were purchased from Cell Signaling Technology. A monoclonal anti-β-actin antibody was purchased from Sigma-Aldrich. Tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat anti-rabbit and anti-mouse IgGs were purchased from the Zhongshan Company. Anti-PEDV rabbit polyclonal antibodies and anti-PEDV N monoclonal antibodies were prepared in our laboratory.

**Plasmids and siRNA.** The NF-κB/luciferase reporter plasmid (pNF-κB-Luc) was kindly provided by Dr Shaobo Xiao (Huazhong Agricultural University, Wuhan, Hubei Province, China). The internal-control plasmid-encoding Renilla luciferase (pRL-TK) was purchased from Promega. The vectors pcDNA3.1(+) and pEGFP-N1 were kept in our laboratory. PEDV (strain CV777; NCBI accession no: AF353511) structural protein genes (including N, M and E) were amplified by PCR using specific primers, and the PCR products were subcloned into the pcDNA3.1 (+) vector. The pcDNA3.1S expression plasmid was constructed in our laboratory. Several deletion mutants of the viral N gene were amplified, using pcDNA3.1N as a template. These mutants were then cloned into pcDNA3.1 (+). The porcine p65 gene (NCBI downloaded from www.microbiologyresearch.org by IP: 54.70.40.11 On: Wed, 03 Apr 2019 21:24:08 1764 Journal of General Virology 96
Transfections and luciferase reporter assays. In 24-well plates, IEC cells (1 x 10^5 cells per well) were co-transfected with 0.2 μg pNF-κB-Luc and 0.1 μg phRL-TK using Lipofectamine 2000 (Invitrogen) reagent, and cells were then infected with PEDV or mock infected. The phRL-TK Renilla luciferase vector was used as an internal control for the dual-luciferase reporter assay. In selected experiments, IECs were co-transfected for 24 h with 0.5 μg of the pEGFP-N1 vector. All primers used in this study are listed in Table 1. Small interfering RNA (siRNA) molecules targeting RIG-I, MyD88, TRIF, TLR2, TLR3, TLR4, TLR8, TLR9 and a scrambled control were obtained from Shanghai GenePharma (Table 2).

**Table 1. Sequences of primers used for cloning the porcine p65 gene, PEDV structural genes and N-protein deletion mutants**

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>p65 sense</td>
<td>5′-CCCAAGCTTATGAGACCCCTTTCCTCCCT3′</td>
</tr>
<tr>
<td>p65 anti-sense</td>
<td>5′-CGGGGTACCCACATCTGTCAGTCAACAGAAG3′</td>
</tr>
<tr>
<td>M sense</td>
<td>5′-CGGGGAATTTCCTGAGGTTTTCAT3′</td>
</tr>
<tr>
<td>M anti-sense</td>
<td>5′-CGGCTCAGGTTAGACTAATAGGACAC3′</td>
</tr>
<tr>
<td>E sense</td>
<td>5′-CCGGGTACCACTGCTTCGATGTATAGAAG3′</td>
</tr>
<tr>
<td>E anti-sense</td>
<td>5′-CCGTCGGGTTATACGTCGTTAGCAGT3′</td>
</tr>
<tr>
<td>N sense*</td>
<td>5′-CCGGGTAGACCATGGCTCTGTCAGTT3′</td>
</tr>
<tr>
<td>N136 sense</td>
<td>5′-CGGGGTACCACTGGAACCTAAACACTCC3′</td>
</tr>
<tr>
<td>N290 sense</td>
<td>5′-CGGGGTACCACTGGAACCTAAACACTCC3′</td>
</tr>
<tr>
<td>N365 sense</td>
<td>5′-CGGGGTACCACTGGAACCTAAACACTCC3′</td>
</tr>
<tr>
<td>N70 anti-sense</td>
<td>5′-CGGCTCGAGGTTAGGTTCAATTGCT3′</td>
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<tr>
<td>N135 anti-sense</td>
<td>5′-CGGCTCGAGGTTAAACATCAACTACAC3′</td>
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<tr>
<td>N289 anti-sense</td>
<td>5′-CGGCTCGAGGTTATTTCGCGCTTGGA3′</td>
</tr>
<tr>
<td>N anti-sense†</td>
<td>5′-CGGCTCGAGGTATACGTCGTTAGCAGT3′</td>
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</table>

*Forward primer for amplifying the N70, N135 and N289 N protein deletion mutants.
†Reverse primer for amplifying the N136, N290 and N365 N protein deletion mutants.

Accession no: EU399817.1 was cloned from porcine kidney cells by performing RT-PCR, using a specific primer pair. The p65 gene was inserted into the pEGFP-N1 vector. All primers used in this study are listed in Table 1. Small interfering RNA (siRNA) molecules targeting RIG-I, MyD88, TRIF, TLR2, TLR3, TLR4, TLR8, TLR9 and a scrambled control were obtained from Shanghai GenePharma (Table 2).

**Immunofluorescence assays.** IECs were seeded into 96-well plates for 24 h and then infected with PEDV at different m.o.i. (0.01, 0.1 or 1) for 48 h or at the same m.o.i. (1) at the time points tested. Infection of PEDV in IECs was detected in indirect IFAs. Briefly, cells were fixed with 4% formaldehyde for 30 min at room temperature, quenched with 0.1 M glycine for 5 min and permeabilized with 0.1% Triton X-100 for 10 min. Cells were then incubated at 37°C for 30 min. Fluorescence signals were visualized using a fluorescence microscope (Leica).

To detect p65 nuclear translocation, IECs were seeded onto 24-well plates at a density of 1 x 10^5 cells per well and grown in monolayers. Cells were then transfected with 1 μg of the p65-GFP fusion expression plasmid using Lipofectamine 2000, followed by PEDV infection at an m.o.i. of 1. Twenty-four hours later, cells were retransfected for 12 h with poly(I:C) as a positive control. To detect the PEDV N-protein-induced p65 nuclear translocation, IECs were co-transfected for 24 h with 0.5 μg of the p65-GFP or pcDNA3.1N expression plasmids, or the empty vectors. Cells were fixed and incubated with rabbit anti-PEDV polyclonal antibody (1:500) or mouse anti-PEDV monoclonal antibody at 37°C for 1 h. After fixation, cells were permeabilized with 0.1% Triton X-100 for 10 min. Cells were then incubated with a TRITC-conjugated goat anti-rabbit IgG (1:200) for 30 min. Fluorescence signals were visualized using a fluorescence microscope (Leica).

**Table 2. Sequences of siRNAs used in this study**

<table>
<thead>
<tr>
<th>Name</th>
<th>Region</th>
<th>Sequence (sense)</th>
<th>Sequence (anti-sense)</th>
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</thead>
<tbody>
<tr>
<td>RIG-1</td>
<td>950–969</td>
<td>5′-GGUACAAAGGGUUUUGACAGGCAGGCUATT-3′</td>
<td>5′-AUGCUGCCACAUUUUGACATT-3′</td>
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<tr>
<td>TRIF</td>
<td>264–285</td>
<td>5′-GGGCGGCUUGCAAUACAAUCUAAAT-3′</td>
<td>5′-UUAGAAGUGUGUAGGAGCCGCTT-3′</td>
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<tr>
<td>MyD88</td>
<td>102–123</td>
<td>5′-GGCGUGUUGCCUCUCAUGCUUAAAT-3′</td>
<td>5′-UUAGAAAGUGGACUGGCAGCCGCTT-3′</td>
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<tr>
<td>TLR2</td>
<td>210–229</td>
<td>5′-CCAGACUUUUGGACGCUUUCAAAT-3′</td>
<td>5′-AUGGAGCUUAAGAAGCUGGT-3′</td>
</tr>
<tr>
<td>TLR3</td>
<td>1069–1088</td>
<td>5′-GCUUAGUGAGUAGUUGCUAAATT-3′</td>
<td>5′-UUUACCAAAUACAGUAAAGCCTT-3′</td>
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<tr>
<td>TLR4</td>
<td>367–386</td>
<td>5′-GCAAUGGAGGCUAAUUCUAAATT-3′</td>
<td>5′-UAAGAAUACUGCUUCAAUGCTT-3′</td>
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<tr>
<td>TLR8</td>
<td>747–766</td>
<td>5′-GCUGUAGAAGACUGAUGAATT-3′</td>
<td>5′-UAACCGUUAGCUGCUUCAAAGCCTT-3′</td>
</tr>
<tr>
<td>TLR9</td>
<td>251–270</td>
<td>5′-GCGCCUCUCUCUACUUCUCCCAAT-3′</td>
<td>5′-UUUAGCAGACGGAGGAGGCCTT-3′</td>
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<tr>
<td>NC</td>
<td></td>
<td>5′-UUUCUCCGAACCUGCAGCUAATT-3′</td>
<td>5′-ACCGAGACCCGAAGAATT-3′</td>
</tr>
</tbody>
</table>
Real-time RT-PCR. IECs were seeded into 24-well plates at a density of 1 × 10^5 cells per well. The interference effects of siRNA molecules in IECs were evaluated by real-time RT-PCR. Briefly, IECs were transfected with siRNAs (140 ng) for 24 h, and total RNA was extracted using TriQuick Reagent (Beijing Solarbio Science & Technology) according to the manufacturer’s instructions. Total RNA (1 µg) was reverse-transcribed into cDNA in 20 µl reaction volumes. cDNAs were then analysed by real-time RT-PCR using specific primer pairs, as described by Song et al. (2013) and Uddin et al. (2013). Real-time RT-PCR was performed using a Roche LightCycler 480 Real-Time PCR System, and relative expression levels were evaluated by the 2^-DeltaDelta method (Livak & Schmittgen, 2001).

Western blot analysis. Nuclear extracts were subjected to Western blot analysis as described by Lee & Kleiboecker (2005). Briefly, cells were harvested with trypsin-EDTA and extracted using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo) according to the manufacturer’s protocols. The concentration of nuclear protein was determined using the Thermo Scientific BCA Protein Assay with BSA as a standard. The samples were separated by 12% SDS-PAGE. Subsequently, proteins were transferred to a nitrocellulose membrane. The membrane was blocked with 5% (w/v) BSA in Tris-buffered saline (10 mM Tris/HC1 at pH 7.5 and 150 mM NaCl) containing 0.05% Tween 20 (TBST) at room temperature for 1 h, then incubated with a rabbit anti-p65, a mouse anti-β-actin or a mouse anti-PEDV N monoclonal antibody (1:1000) at 4 °C overnight. The membranes were washed three times with TBST and incubated with an HRP-conjugated goat anti-rabbit or goat anti-mouse IgG antibody (1:2500) at room temperature for 1 h. After washing, proteins were detected using an Enhanced Chemiluminescence (ECL) Detection System (Clinx Science Instruments).
PEDV activates NF-κB in IECs


