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Veterinary Microbiology 274 (2022) 109553

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Porcine deltacoronavirus E protein induces interleukin-8 production *via* NF-κB and AP-1 activation

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ARTICLE INFO

Keywords:
PDCoV
E
IL-8
Inflammation

ABSTRACT

Infection induces the production of proinflammatory cytokines and chemokines such as interleukin-8 (IL-8) and interleukin-6 (IL-6). Although they facilitate local antiviral immunity, their excessive release leads to life-threatening cytokine release syndrome, exemplified by the severe cases of coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection. In the present study, we found that interleukin-8 (IL-8) was upregulated by PDCoV infection. We then demonstrated that PDCoV E protein induced IL-8 production and that the TM domain and the C-terminal domain of the E protein were important for IL-8 production. Subsequently, we showed here that deleting the AP-1 and NF-κB binding motif in porcine IL-8 promoter abrogated its activation, suggesting that IL-8 expression was dependent on AP-1 and NF-κB. Furthermore, PDCoV E induced IL-8 production, which was also dependent on the NF-κB pathway through activating nuclear factor p65 phosphorylation and NF-κB inhibitor alpha (IκBα) protein phosphorylation, as well as inducing the nuclear translocation of p65, eventually resulting in the promotion of IL-8 production. PDCoV E also activated c-fos and c-jun, both of which are members of the AP-1 family. These findings provide new insights into the molecular mechanisms of PDCoV-induced IL-8 production and help us further understand the pathogenesis of PDCoV infection.

1. Introduction

Since the start of this century, three animal coronaviruses have crossed the species barrier and caused severe disease in humans. In 2003, the severe acute respiratory syndrome coronavirus (SARS-CoV) that originated in bats caused the SARS outbreak and plunged the world into panic (Drosten et al., 2003; Ksiazek et al., 2003). Then the Middle East respiratory syndrome coronavirus (MERS-CoV) emerged in 2012 and caused two regional outbreaks with intermittent sporadic cases (Zaki et al., 2012; Azhar et al., 2014). The ongoing coronavirus disease 2019 (COVID-19) pandemic is caused by SARS-CoV-2 and has become the most devastating outbreak since the H1N1 influenza in 1918 (Jiang et al., 2020; Li et al., 2020). Therefore, unraveling the mechanisms of coronavirus pathogenesis is a pressing problem with great clinical importance.

Porcine deltacoronavirus (PDCoV) is a recently discovered enteropathogenic coronavirus and has caused significant economic impacts on the pork industry (Jung et al., 2016; Xu et al., 2020; Zhou et al., 2021). PDCoV, similar to other swine enteric coronaviruses, including transmissible gastroenteritis virus (TGEV) and porcine epidemic diarrhea virus (PEDV), has caused frequent occurrences of diarrhea, vomiting, and dehydration in piglets (Wang et al., 2018; Zhu et al., 2018; Zhang et al., 2019; Stoian et al., 2020; Yin et al., 2020; Zhou et al., 2021). Clinically, PDCoV infection commonly occurs in the form of co-infection with PEDV or TGEV, which has caused significant economic losses within the global swine industry (Lednicky et al., 2021b). More recently, Lednicky et al. reported for the first time that PDCoV was detected in plasma samples of three Haitian children with acute undifferentiated febrile illness, suggesting that PDCoV may have jumped from pigs to humans, highlighting the significant threat to human health posed by this emerging CoV and attracting tremendous attention to the topic (He et al., 2020; Lednicky et al., 2021a), because CoVs have

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https://doi.org/10.1016/j.vetmic.2022.109553
Received 27 May 2022; Received in revised form 22 August 2022; Accepted 27 August 2022
Available online 31 August 2022

repeatedly crossed the host barrier between different animals, like swine acute diarrhea syndrome coronavirus from bats to swine (He et al., 2022) and from an animal reservoir to humans for the ongoing SARS-CoV-2 pandemic (Zhou et al., 2020).

The innate immune system is necessary for the initial detection and restriction of viral infections, as well as the subsequent activation of the adaptive immune response. Coronaviruses are recognized by cytosolic and/or endosomal pattern recognition receptors (PRRs), which activate adapter proteins and downstream pathways. This eventually leads to the activation of critical transcription factors such as nuclear factor kappa light chain enhancer of activated B cells (NF-κB), interferon regulatory factor 3/7 (IRF-3/7), and activator protein 1 (AP-1) (Le Bon and Tough, 2002; Taniguchi and Takaoka, 2002; Perry et al., 2005; Lin et al., 2016; Li et al., 2017; Fang et al., 2018). These proteins then activate the transcription of type I/III interferons (IFN-I/III) and proinflammatory cytokines/chemokines such as tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6), and interleukin-8 (IL-8). Acting locally, these cytokines/chemokines recruit immune cells and facilitate antiviral responses; but their excessive and uncontrolled release can lead to life-threatening cytokine release syndrome (CRS) that underlies the pathogenesis of severe coronavirus diseases (Chua et al., 2020; Sallenave and Guillot, 2020; Gustine and Jones, 2021). Indeed, multiple proinflammatory cytokines have been implicated in the pathogenesis of severe COVID-19 (Cao, 2020). Among them, high levels of IL-6 and IL-8 were observed in patients with severe or critical COVID-19, correlated with lymphocytopenia that was predictive of disease progression (Zhang et al., 2020). High levels of IL-6 and IL-8 were also detected in SARS patients (Zhang et al., 2004; Huang et al., 2005; Yu et al., 2005) and in some cell lines infected with SARS-CoV (Law et al., 2005; Spiegel and Weber, 2006). However, it is still unclear whether acute PDCoV infection induces pro-inflammatory cytokine immune responses.

In the present study, we demonstrated that PDCoV infection induced IL-8 upregulation. We then demonstrated that PDCoV E protein induced IL-8 production and the TM domain and the C-terminal domain of the E protein were important for IL-8 production. Subsequently, we showed here that deleting the AP-1 and NF-κB binding motif in the porcine IL-8 promoter abrogated its activation, suggesting that IL-8 expression was dependent on AP-1 and NF-κB. Furthermore, NF-κB and AP-1 activation were crucial in IL-8 expression stimulated by PDCoV E. Our study provides further information for our better understanding of PDCoV pathogenesis.

2. Materials and methods

2.1. Cell culture and viruses

IPEC-J2 cells (porcine small intestine epithelial cell clone J2, donated by Yanming Zhang of Northwest A&F University, China) (Liu et al., 2010), ST cells (swine testicular cells; ATCC) and HEK293T cells (human embryonic kidney epithelial cells; ATCC) were cultured in Dulbecco's minimum essential medium (DMEM) (Life Technologies, USA) supplemented with 10 % heat-inactivated fetal bovine serum (FBS) (Gibco, USA), 100 U/ml penicillin, 100 μg/ml streptomycin in an incubator with 5 % CO_2 at 37 °C (Thermo Scientific, USA). PDCoV strain NH (GenBank: KU981062.1) was prepared and titrated as previously described (Tang et al., 2022).

2.2. Primers and antibodies

The primer sequences are listed in Table 1. All plasmid constructs were confirmed by sequencing. The listed antibodies were used in this study including anti-p65 primary antibody (AnaSpec, USA) and anti-phospho-p65, anti- phospho-IκBα, and anti-β-actin primary antibodies (Cell Signaling Technology, USA); and anti-phospho-c-Fos, anti-phospho-c-Jun, anti-c-Fos and anti-c-Jun (Abcam, UK). Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse or anti-rabbit IgG and horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit IgG antibodies were purchased from Beyotime Biotechnology (Beyotime, China).

2.3. Virus infection

IPEC-J2 or ST cells monolayers were infected with PDCoV for 1 h at 37 °C. Unbound virus was removed, and cells were maintained in complete medium for various time points until samples were harvested.

2.4. Transfection

Cells were transfected with the indicated plasmids using X-tremeGENE transfection reagent according to the manufacturer's instructions (Roche, USA). At the indicated times, cell samples were collected and lysed in RIPA buffer (Beyotime, Nantong, China) for the western blot analysis of target proteins.

2.5. Immunofluorescence assay (IFA)

IFA was performed as described previously with slight modifications (Guo et al., 2016). Briefly, HEK293T cells were either vector-transfected or transfected with pCAGGS-E for 30 h, followed by staining with specific monoclonal antibodies for one hour. After the removal of unbound antibodies, the cells were stained with a secondary antibody for another hour, followed by nuclei staining with DAPI (4,6-diamidino-2-phenylindole; Sigma). After washing the cells, the fluorescence was visualized with confocal laser scanning microscopy.

2.6. Western blot

Western blot analysis was performed as previously described (Luo et al., 2017). Treated samples were lysed in RIPA buffer containing protease inhibitor cocktail and phosphatase inhibitors (Roche, Switzerland) and separated by SDS-PAGE under reducing conditions and transferred onto a PVDF membrane (Merck Millipore, USA). After

Table 1
Primers used in this study.

Primer	Forward (5′→3′)	Reverse (5′→3′)
qIL-8	CCACACCTTTCCACCCCAAA	TTGTTGCTTCTCAGTTCTCTTCA
qGAPDH	CCTTCCGTGTCCCTACTGCCAAC	GACGCCTGCTTCACCACCTTCT
IL-8 F0	GAGAGCAGTAATCTCTCCTGG	GAAGGCAACAGCCAGTITGGAAGT
IL-8 F1	GCTCAATGCTGCTGAAAACA	GAAGGCAACAGCCAGTITGGAAGT
IL-8 F2	CCAATCATTAGAGGAGTCAG	GAAGGCAACAGCCAGTITGGAAGT
IL-8 F3	GATGGTTGCG TAGTGTGGAAT	GAAGGCAACAGCCAGTITGGAAGT
IL-8 F4	GCACATGTTCCCTACTCTTG	GAAGGCAACAGCCAGTITGGAAGT
pCAGGS-S	TTAAGGATTCGAATGCAGAGGGCCCTGCTGAT	TATACTCGAGCCACTCCTTGAACT
pCAGGS-E	CGGCTAGCATGGTGGTGGATGATTG	CGTCTAGACACATAATGGGTGTTGCG
pCAGGS-M	CGGCTAGCATGAGCGATGCGGAAGAATG	CGTCTAGACATATATTTATACAGGCGCG
pCAGGS-N	CGCGGATCCATGGCCGCACCAGTAGTCCCTACTA	CCGCTCGAGCGCTGCTGATTCCTGCTTTATCTCA

blocking, the membranes were incubated with a primary antibody and then probed with an appropriate IRDye-conjugated secondary antibody (Li-Cor Biosciences, Lincoln, NE). The membranes were scanned using an Odyssey instrument (Li-Cor Biosciences) according to the manufacturer's instructions.

2.7. Quantitative RT-PCR

Quantitative RT-PCR analysis was carried out as described previously (Guo et al., 2014). The total RNA was extracted from cells and subjected to quantitative RT-PCR using specific primers as listed in Table 1. Relative gene quantification was performed using the 2(-Delta Delta C(T)) method (Livak and Schmittgen, 2001).

2.8. Dual luciferase reporter assay

HEK293T cells were cotransfected with 0.1 μg of porcine IL-8 promoter F2 vector, 0.004 μg pRL-TK (Promega Biotech Co., Ltd, Beijing, China) and 0.4 μg of pCAGGS/S/E/M/N-HA by using X-tremeGENE reagents (Promega, Beijing, China). Meanwhile, 0.1 μg of porcine IL-8

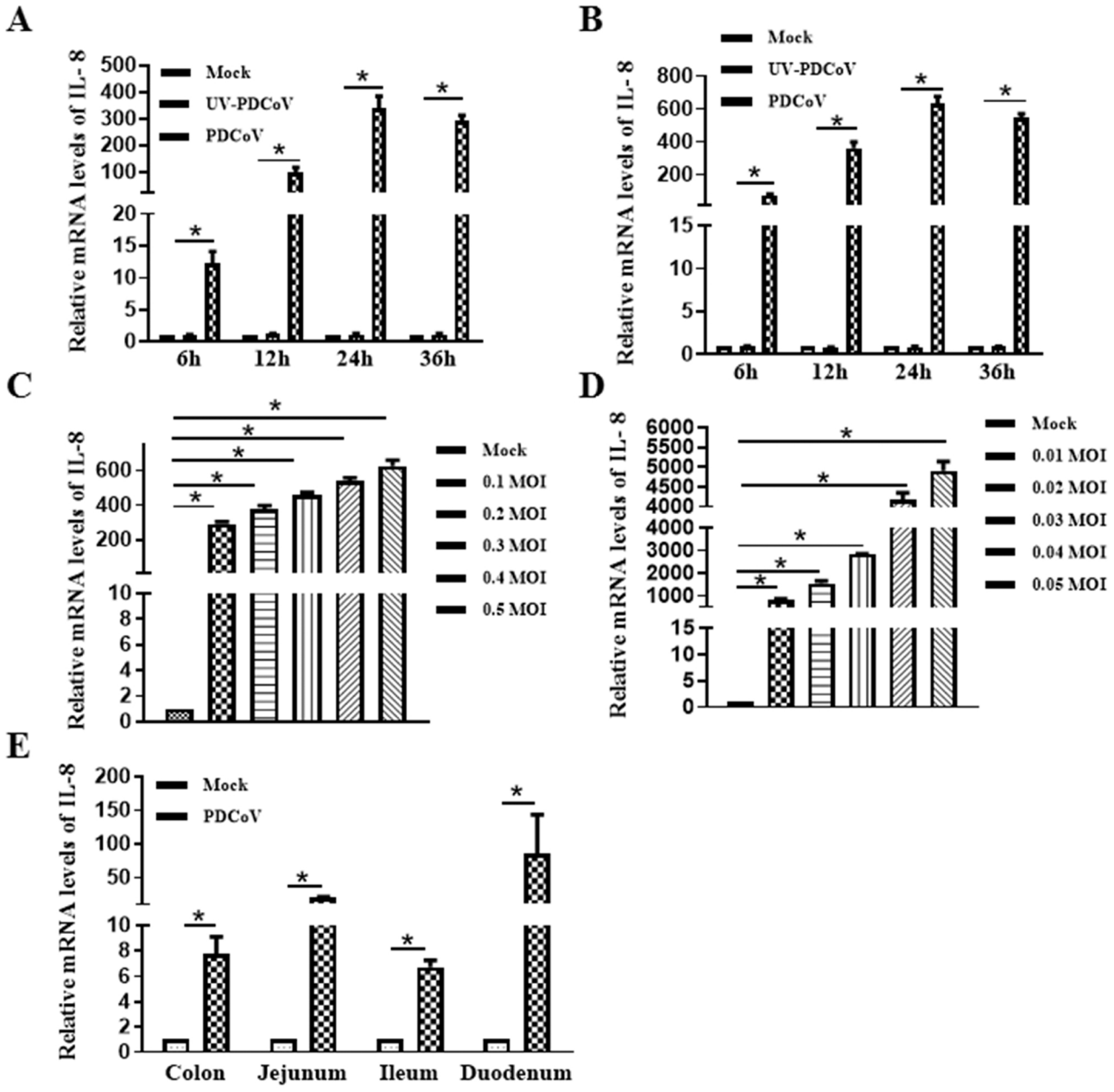

Fig. 1. IL-8 was upregulated by PDCoV infection. A, IPEC-J2 cells were inoculated with medium alone, PDCoV, or UV-inactivated PDCoV at an MOI of 0.1. Total RNA was extracted from cell lysates at 6, 12, 24, and 36 h post-inoculation. quantitative RT-PCR was used to analyze IL-8 expression; B, ST cells were inoculated with medium alone, PDCoV, or UV-inactivated PDCoV at an MOI of 0.01. Total RNA was extracted from cell lysates at 6, 12, 24, and 36 h post-inoculation. quantitative RT-PCR was used to analyze IL-8 expression; C, IPEC-J2 cells were either mock infected or infected with PDCoV at MOIs of 0.1, 0.2, 0.3, 0.4, and 0.5 for 24 h, and total RNA was extracted for detection by quantitative RT-PCR; D, ST cells were either mock infected or infected with PDCoV at MOIs of 0.01, 0.02, 0.03, 0.04, and 0.05 for 24 h, and total RNA was extracted for detection by quantitative RT-PCR; E, Pigs were infected intranasally with 2 ml (10^5 $TCID_{50}$ virus/ml) PDCoV. Samples were collected at 2 days post infection. IL-8 mRNA was quantified by quantitative RT-PCR. And results were normalized to GAPDH and expressed as fold induction over samples from uninfected pigs. The results are representative of three independent experiments (the means ± SD). * $P < 0.05$. The P value was calculated using Student's t-tests.

promoter F2 vector, 0.004 μg pRL-TK, and 0.4 μg of pCAGGS-HA were also cotransfected as a control group. At 30 h post-transfection, the cell lysates were prepared, and dual luciferase reporter assays were carried out in a GloMax 96 microplate luminometer (Promega, Beijing, China) using a Dual-Glo luciferase kit (Promega, Beijing, China) according to the manufacturer's instructions.

2.9. ELISA

The IL-8 protein levels in plasma and cell culture supernatants were measured using porcine IL-8 ELISA kits (RD SYSTEMS) in accordance with the manufacturer's instructions.

2.10. Animal experiment

Four specific-pathogen-free (SPF) pigs were randomly assigned to 2 experimental groups: the infected group 1 (n = 2) and the uninfected group 2 (n = 2). The two different groups were challenged orally with PDCoV NH (2 ml of 10^5 TCID50/ml virus stock per pig), and DMEM served as the mock control. All the pigs were euthanized for pathological examination when the onset of clinical signs was observed. Fresh samples, including colon, duodenum, jejunum and ileum, were collected during the necropsy. The fresh samples and the piglet serum were collected for IL-8 detection.

2.11. Statistical analysis

Variables are expressed as mean ± S.D. Statistical analyzes were performed using the student's *t* test. A p value of < 0.05 was considered significant.

3. Results

3.1. PDCoV induces IL-8 production both in vitro and in vivo

To investigate whether PDCoV infection can induce IL-8 production, IPEC-J2 and ST cells were used to conduct a PDCoV infection assay *in vitro*. We infected IPEC-J2 and ST cells with PDCoV and examined IL-8 expression by quantitative RT-PCR at the indicated times post infection. Our results showed that PDCoV infected IPEC-J2 cells upregulated IL-8 mRNA expression by about 13, 97, 340, and 300 folds compared to the uninfected controls at 6, 12, 24, and 36 h post infection (hpi), respectively (Fig. 1A). In PDCoV-infected ST cells, the IL-8 mRNA level was significantly elevated by about 70, 370, 610, and 580 folds compared to the uninfected controls at 6, 12, 24, and 36 hpi, respectively (Fig. 1B). The upregulation of IL-8 by PDCoV infection could be caused by invading virions or by intermediate products generated during virus replication. To investigate whether viral replication is required for upregulation of IL-8, PDCoV virions were inactivated by UV irradiation. Cells were incubated with the same amounts of UV-inactivated viruses, and no induction of IL-8 was observed in cells incubated with UV-PDCoV (Fig. 1A and B). The upregulation of IL-8 was in a dose-dependent manner (Fig. 1C and D). Collectively, these data indicate that PDCoV infection remarkably induces IL-8 production *in vitro*.

To investigate whether PDCoV has the ability to induce IL-8 production *in vivo*, SPF pigs were orally infected with the PDCoV strain, and samples were collected at 2 days post infection for IL-8 analysis. The intestinal tissues collected from these piglets were analyzed by quantitative RT-PCR for IL-8 analysis. Our results showed that IL-8 mRNA expression was significantly induced in intestinal tissues from PDCoV-infected piglets (Fig. 1E). Collectively, these data indicate that PDCoV infection remarkably induces IL-8 production both *in vivo and in vitro*.

3.2. PDCoV induces IL-8 protein expression

The expression of the IL-8 protein was also determined. The secretion of IL-8 in the supernatant of the uninfected and infected cells was examined using an ELISA method. As shown in Fig. 2A, PDCoV-infected IPEC-J2 cells were found to express higher levels of IL-8 compared to the uninfected cells. A similar induction of IL-8 expression was also observed in PDCoV-infected ST cells (Fig. 2B). Compared with the uninfected piglets, the serum levels of IL-8 were significantly upregulated in PDCoV-infected piglets (Fig. 2C). In summary, these findings suggest that PDCoV infection remarkably upregulates IL-8 production both *in vivo and in vitro*.

3.3. PDCoV E protein upregulates IL-8 expression

To study the mechanisms underlying the transcriptional regulation of PDCoV-induced IL-8 production, we cloned a 2000-bp fragment of the 5′- flanking region of the porcine IL-8 gene. To evaluate the porcine IL-8 promoter activity and to determine the functional domain responsive to PDCoV infection, pGL3 luciferase reporter plasmids encoding a series of truncated deletions were constructed and schematically shown in Fig. 3A. HEK 293 cells transfected with these constructs were then infected with or without PDCoV infection. The luciferase assay showed that all the constructs, except the construct IL-8 F3 (−241/+26-luc) and IL-8 F4 (−140/+26-luc), exhibited higher luciferase activities after PDCoV infection. Among them, IL-8 F2 (−271/+26-luc) was more efficiently activated by PDCoV, which manifested a 450 fold induction over its basal-level activity (Fig. 3B). This observation suggests that the region from positions − 271 to + 26 in the porcine IL-8 promoter is sufficient for PDCoV-induced promoter activity and that the regulatory elements might exist in this region.

To determine which PDCoV structural proteins could activate the IL-8 promoter to upregulate the mRNA expression levels of the IL-8 genes by using a luciferase reporter assay. HEK293T cells were cotransfected with IL-8 F2 (−271/+26-luc), pRL-TK and pCAGGS-S/E/M/N-HA, respectively, or along with pCAGGS-HA. At 30 h post-transfection, the luciferase activity was detected. The results indicated that E significantly enhanced IL-8 promoter activity (Fig. 3C). To further validate this, HEK293T cells were transfected with IL-8 F2 (−271/+26-luc), along with pRL-TK and different amounts of the E expression plasmid, and the luciferase activity was measured at 30 h post-transfection. The data clearly showed that overexpression of E was responsible for the activation of the IL-8 promoter activity in a dose dependent manner (Fig. 3D).

3.4. Functional domain of the E protein responsible for IL-8 upregulation

To determine which domain of E is important for IL-8 production, using the bioinformatics approach (https://www.ebi.ac.uk/Tools/msa/clustalo), PDCoV E, an 83-amino-acid protein, is usually divided into three domains: the N-terminal domain, the TM domain and the C-terminal domain (Fig. 4A). We constructed three truncated mutants, including E△N, E△TM, and E△C (Fig. 4B). Each of the mutant vectors was transfected into HEK293T cells, and then IL-8 expression was analyzed. Our results showed that E△TM and E△C remarkably suppressed IL-8 production compared to the full length E protein (Fig. 4C), implying that the TM domain and the C-terminal domain of E were more important for IL-8 production than the N-terminal domain of E.

3.5. AP-1 and NF-κB are critical for IL-8 expression

To gain further knowledge of the transcriptional regulation mechanism of E-induced IL-8 production, using the bioinformatics approach (http://www.cbrc.jp/research/db/TFSEARCH.html), we found that there were several putative transcriptional regulatory elements located in this region, including c/EBP β (−185 to −180), NF-κB (−174 to −166), and AP-1 (−220 to −214) binding sites. To determine which transcriptional regulatory element(s) in this region is important for the activation of the IL-8 promoter by PDCoV E protein, we deleted each of

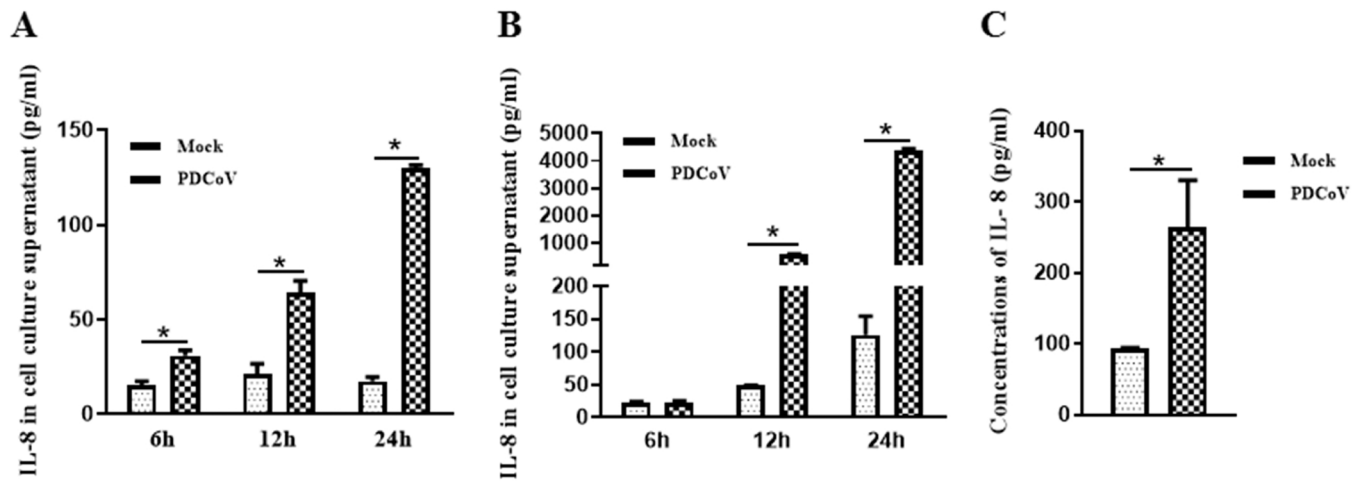

Fig. 2. PDCoV induces IL-8 expression *in vitro and in vivo*. A, IPEC-J2 supernatants were harvested at 6, 12, and 24 hpi post PDCoV infection (MOI=0.1), and levels of IL-8 (pg/ml) released were determined by ELISA; B, ST supernatants were harvested at 6, 12, and 24 hpi post PDCoV infection (MOI=0.01), and levels of IL-8 (pg/ml) released were determined by ELISA; C, The concentrations of IL-8 in serums collected from piglets post-challenge were detected *via* ELISA assay. The data are representative of three independent experiments (the means ± SD). * $P < 0.05$. The P value was calculated using Student's t-tests.

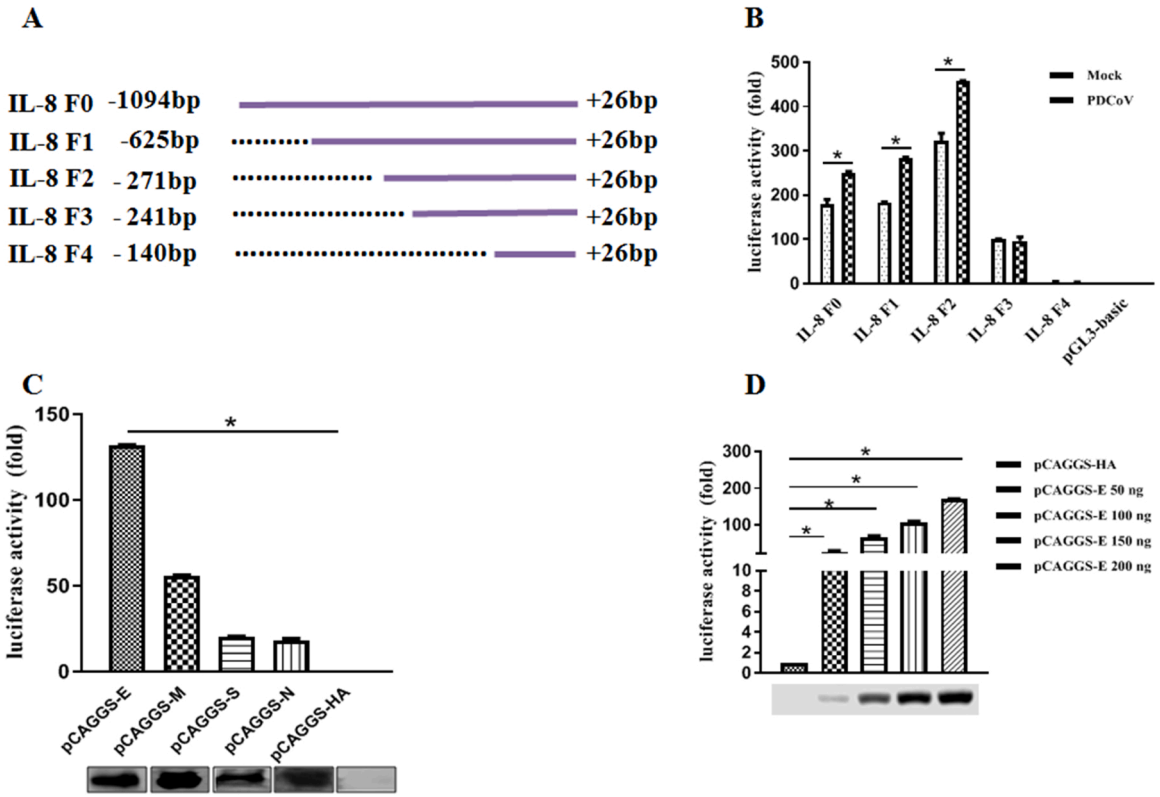

Fig. 3. PDCoV E up-regulates IL-8 expression. A, Cloning and sequence analysis of the 2000-bp porcine IL-8 promoter. The positions of the putative regulatory motifs are relative to the transcription initiation site. Schematic representation of the porcine IL-8 promoter and promoter deletion mutants inserted into pGL3 basic luciferase vectors: IL-8 F0 (−1094/+26-luc), IL-8 F1 (−625/+26-luc), IL-8 F2 (−271/+26-luc), IL-8 F3 (−241/+26-luc), IL-8 F4 (−140/+26-luc); B, The porcine IL-8 promoter vectors or pGL3 basic empty vector were transfected into HEK 293 cells. Twenty-four hours later, cells were inoculated with PDCoV (MOI=0.1) or medium. Cells were harvested to determine luciferase activity at 24 hpi.; C, HEK293T cells were cotransfected with porcine IL-8 F2 (−271/+26-luc) vector, together with pRL-TK, and plasmids encoding the indicated PDCoV structural protein or pCAGGS-HA empty vector as negative control. At 30 h post-transfection, cells were harvested and analyzed by western blotting using the anti-HA antibody and cell extracts were prepared for luciferase reporter gene assays; D, HEK293T cells were cotransfected with porcine IL-8 F2 (−271/+26-luc) vector, pRL-TK, and the indicated amounts of E expression plasmid, Total amounts of transfected DNA were kept equal by adding empty vector. Cells were harvested and analyzed by western blotting using the anti-HA antibody and luciferase activity measurement at 30 h post-transfection. The results are representative of three independent experiments (the means ± SD). * $P < 0.05$. The P value was calculated using Student's t-tests.

c/EBP β, NF-κB and AP-1 binding sites from IL-8 F2 (−271/+26-luc) to generate differential mutation vectors (Fig. 5A). Thereafter, we monitored the IL-8 promoter activity following transfection with the pCAGGS-E plasmid. The luciferase results showed that there was no significant difference in luciferase activity when the c/EBP β binding site was deleted, whereas mutations with AP-1 or NF-κB binding site

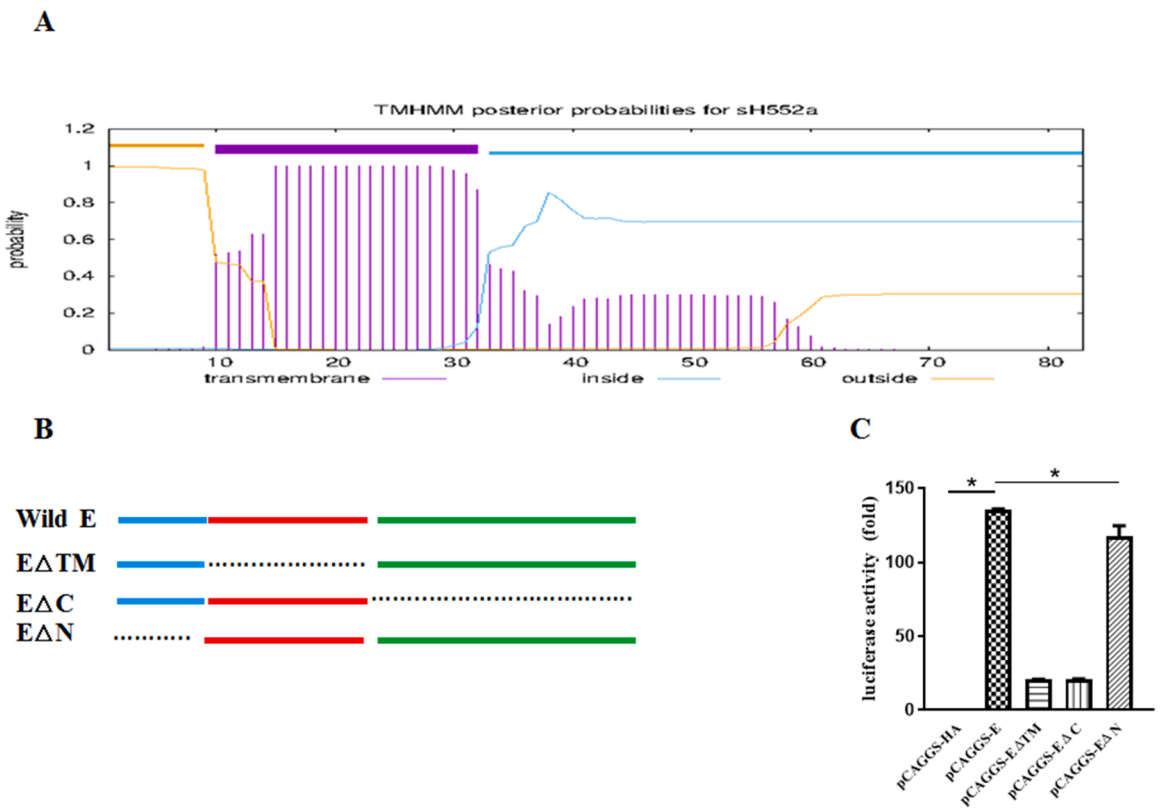

Fig. 4. TM domain and C-terminal domain of E are the key amino acids for IL-8 regulation. A, Schematic representation of PDCoV E protein structure prediction; B, Schematic representation of the wild-type (WT) E and its truncated mutants. The mutants included E△N (amino acids [aa] 10–83), E△TM (amino acids [aa] 1–9 and amino acids [aa] 33–83), and E△C(amino acids [aa] 1–32); C, HEK293T cells were cotransfected with porcine IL-8 F2 (−271/+26-luc) vector, together with pRL-TK, and wild-type E or its truncated mutants or pCAGGS-HA empty vector as negative control. Cells were harvested at 30 h post-infection and assayed for luciferase activity. The results are representative of three independent experiments (the means ± SD). * $P < 0.05$. The P value was calculated using Student's t-tests.

deletion exhibited reduced IL-8 promoter activation. Remarkably, the IL-8 luciferase promoter nearly lost its ability to respond to PDCoV E stimulation when all c/EBP β, AP-1, and NF-κB binding sites were deleted (Fig. 5B), implying that the AP-1 and NF-κB response elements might be critical for PDCoV to activate the IL-8 promoter.

3.6. PDCoV E protein upregulates IL-8 expression by activating NF-κB and AP-1 signaling

NF-κB is an important transcription factor and plays a vital role in regulating the expression of multiple pro-inflammatory cytokines and chemokines. Therefore, to understand the potential roles of PDCoV E in NF-κB signaling, we next verified whether E modulated p65 expression and its phosphorylation. HEK293T cells were either vector-transfected or transfected with pCAGGS-E for 30 h, and then cell lysates were harvested and analyzed through Western blot using specific primary monoclonal antibodies. The results showed that PDCoV E significantly activated the expression of p-p65 and p-IκBα, compared with that in vector-transfected HEK293T cells (Fig. 6A).

The activation of NF-κB is usually characterized by phosphorylation of NF-κB subunit p65 and subsequent nuclear translocation, where it binds to target sequences and initiates inflammatory transcription factors. To understand the basis for PDCoV E upregulation of pro-inflammatory cytokines, we next determined whether E influenced the NF-κB p65 nuclear translocation. HEK293T cells were either vector-transfected or transfected with pCAGGS-E for 30 h, followed by staining with specific monoclonal antibodies, and were observed with a confocal microscope. The results showed that, as expected, p65 was distributed in the cytoplasm in vector-transfected cells (Fig. 6B). In contrast, a large amount of p65 was translocated to the nucleus when transfected with pCAGGS-E. Taken together, these results suggest that PDCoV E protein induces NF-κB p65 phosphorylation and subsequent nuclear translocation.

The role of transcription factor AP-1 in PDCoV E-stimulated IL-8 was determined. HEK293T cells were either vector-transfected or transfected with pCAGGS-E for 30 h, and then cell lysates were harvested and analyzed through Western blot using specific primary monoclonal antibodies. The results showed that PDCoV E activated the phosphorylation of c-Fos and c-Jun, both of which are members of the AP-1 family (Fig. 6C). Our results demonstrate that NF-κB and AP-1 activation are crucial in IL-8 expression stimulated by PDCoV.

4. Discussion

The innate immune system is the host's first line of defense against pathogens, but the excessive production of proinflammatory cytokines and chemokines is considered to be the main mediator in the pathogenesis of coronaviruses (Stark et al., 1998; Scutigliani and Kikkert, 2017; Chathuranga et al., 2021; Zhang et al., 2022). Under different virus infections, the most significantly increased cytokines are usually different. And there is often a difference between the mild and the severe symptoms of the same virus infection. Therefore, the kind of cytokine antagonists that should be used to control the inflammatory responses depends on individual situations. Many previous studies focused on IL-6, which may contribute to disease exacerbation, and some therapeutic approaches based on anti-IL-6 biologics have been proposed (Copaescu et al., 2020; Gubernatorova et al., 2020) and validated (Masia et al., 2020). Given the important significance of IL-8 in the pathology of virus infection , we dissected the underlying mechanism of IL-8 expression under PDCoV infection. In this study, we investigated how PDCoV

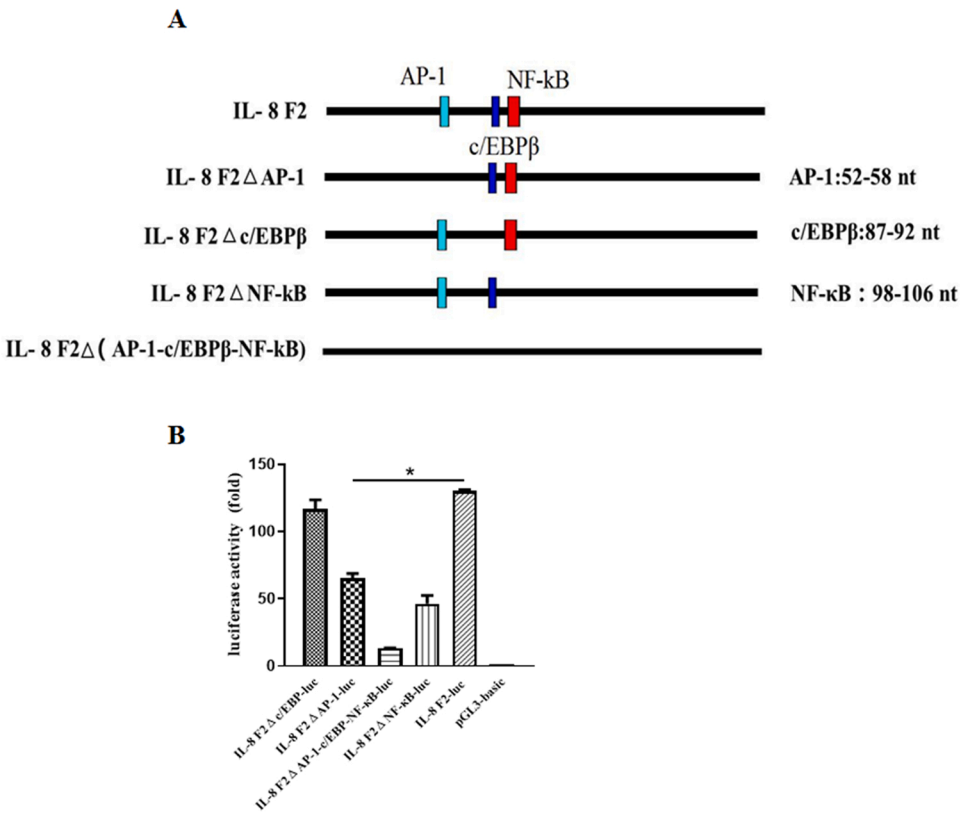

Fig. 5. AP-1 and NF-κB are required for E-induced IL-8 expression. A, Schematic representation of the − 271/+ 26-luc IL-8 promoter deletion mutant constructs including − 271/+ 26(△AP-1)-luc, − 271/+ 26(△NF-κB)-luc, − 271/+ 26(△c/EBP β)-luc, − 271/+ 26 (△AP-1- c/EBP β- NF-κB)-luc; B, HEK293T cells were transfected with the − 271/+ 26-luc IL-8 promoter deletion mutant vectors or pGL3 basic empty vector, together with pRL-TK, and plasmids encoding the PDCoV E protein. The cells were harvested to determine the luciferase activity at 30 h after transfection. The results are representative of three independent experiments (the means ± SD). * $P < 0.05$. The P value was calculated using Student's t-tests.

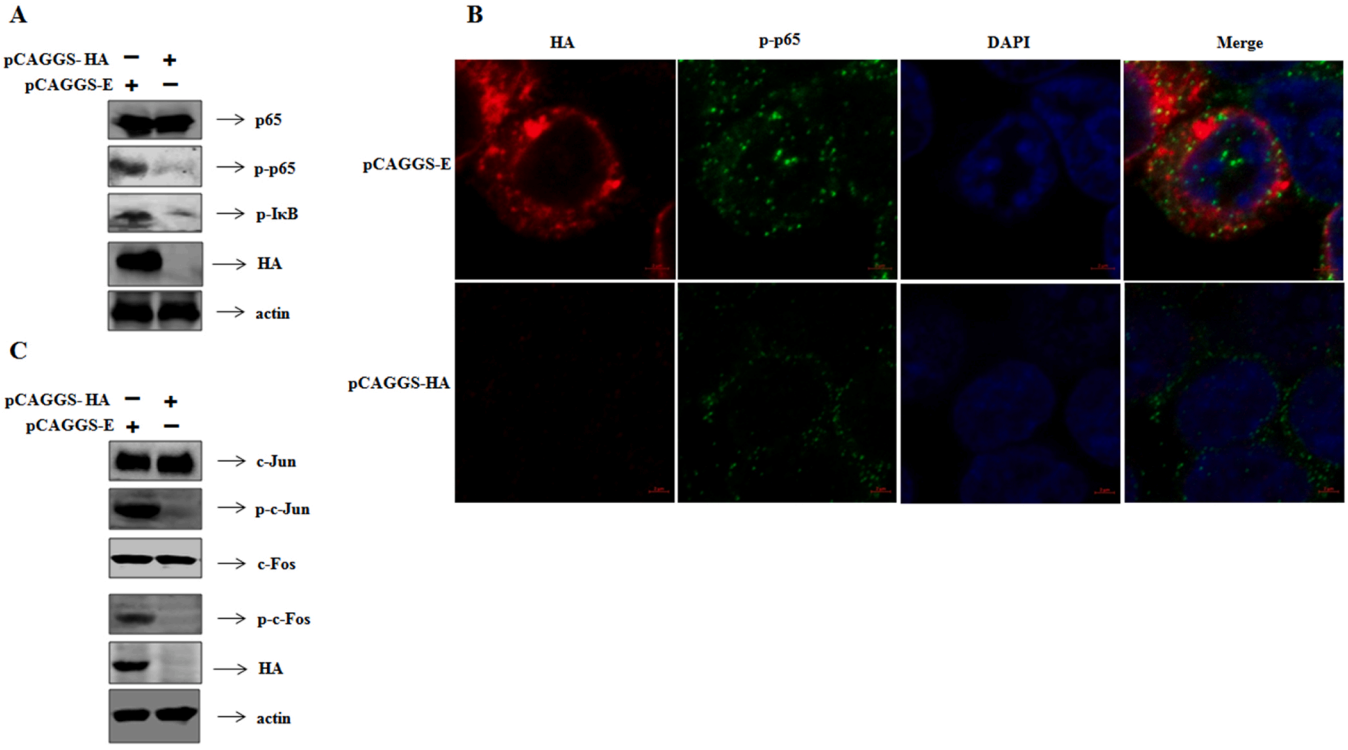

Fig. 6. NF-κB and AP-1 activation are crucial in IL-8 expression stimulated by PDCoV E. A, After HEK293T cells were mock-transfected or transfected with pCAGGS-E for 30 h, and the amount of endogenous phosphop65, p65 and p-IκBα were determined using corresponding specific monoclonal antibodies; B, After HEK293T cells were mock-transfected or transfected with pCAGGS-E for 30 h (red). Cells were fixed and incubated with mouse anti- p65 monoclonal antibody (green) for immunofluorescence assay under confocal laser scanning microscopy. Cellular nuclei were counterstained with DAPI (blue); C, After HEK293T cells were mock-transfected or transfected with pCAGGS-E for 30 h, and cell lysates were analyzed using the antibodies against phospho-c-Fos, phospho-c-Jun. c-Fos and c-Jun.

induced IL-8 production. We showed that PDCoV induced IL-8 production both *in vitro and in vivo*. Several studies have confirmed that virus infection can disturb the expression of cytokines, including the pro-inflammatory cytokines and lead to further tissue damage of the organisms. It was demonstrated that IPEC-J2 cell culture-passaged PDCoV (OH-FD22-P8-I-P4) was enteropathogenic in 10-day-old gnotobiotic pigs, and induced systemic innate and pro-inflammatory cytokine responses during the acute PDCoV infection (Jung et al., 2018). This observation is similar to the increased serum innate (IFNα and IL-22) and the pro-inflammatory (IL-6, TNFα, and IL-12) cytokine responses of 9–10-day old, gnotobiotic or conventional pigs to acute PEDV infection (Annamalai et al., 2015). The pro-inflammatory cytokines of IL-6, IL-8, and TNF-α productions were significantly upregulated in PDCoV-PEDV co-inoculated piglets when compared with the inoculated alone and the control piglets at 3 dpi (Zhang et al., 2021). These data suggest that the infection of PDCoV or PEDV has caused the excessive secretion of pro-inflammatory cytokines (IL-6, IL-8 and TNF-α) and further led to small intestine pathological lesions.

In human and mouse IL-8 promoters, a sequence from nt + 1 to− 133 within the 5′ flanking region of the IL-8 gene is essential and sufficient for the transcriptional regulation of the gene (Mukaida and Murayama, 1998). Analysis demonstrates that the promoter elements contain NF-κB, AP-1, and c/EBP β binding sites (Matsusaka et al., 1993; Mukaida et al., 1998; Nourbakhsh et al., 2001). The promoter is regulated in a cell type-specific fashion, requiring an NF-κB element plus either an AP-1 or a c/EBP β element (Wu et al., 1997; Chang et al., 2004). We cloned the 2000 bp-sequence in the 5′ flanking region of the porcine IL-8 gene. Truncation mutations indicated that the region from − 271 to + 26 bp was essential for the porcine IL-8 promoter activity. However, the longer promoter was not stimulated by PDCoV. The possible reason is that there might be some negative regulation elements in the upstream of the − 271 to + 26 bp. Analysis showed that AP-1, c/EBP β, and NF-κB elements existed in the region from − 271 to + 26 bp. Deletion of these elements suggested that AP-1 and NF-κB elements were the most significant ones in PDCoV induced IL-8 expression.

We find that IL-8 is induced by PDCoV E through the NF-κB and AP-1 pathway. Previous studies have confirmed that PEDV E protein induced ER stress and significantly activated NF-κB which consequently caused the promotion of IL-8 expression (Xu et al., 2013). Recently, the accessory protein ORF3 of PEDV inhibits cellular IL-6 and IL-8 production by blocking the NF-κB p65 activation (Wu et al., 2020). However, the mechanisms underlying the transcriptional regulation of PDCoV-induced IL-8 production is still unclear. In our study, we demonstrated that PDCoV E induced IL-8 expression and that the TM domain and the C-terminal domain in E were essential for the production. Further research suggests that NF-κB and AP-1 activation are crucial in IL-8 expression stimulated by PDCoV E.

5. Conclusion

In summary, our results showed that PDCoV infection induced IL-8 upregulation both *in vitro and in vivo*. We then demonstrated that PDCoV E protein induced IL-8 production and the TM domain and the C-terminal domain of the E protein were important for IL-8 production. Subsequently, we showed here that deleting the AP-1 and NF-κB binding motif in the porcine IL-8 promoter abrogated its activation, suggesting that IL-8 expression was dependent on AP-1 and NF-κB. Furthermore, PDCoV E induced IL-8 production, which was also dependent on the NF-κB and AP-1 pathway. These findings expanded our understanding of the complex mechanisms regulating the innate immune response during PDCoV infection and provided new insights into the immunopathologies associated with severe coronavirus diseases.

CRediT authorship contribution statement

Conceptualization, Y.W.; methodology, H.Z., M.L., Y.Z. and Z.S.; writing, J.C., H.S. and L.G.; supervision, D.S. and L.F. All authors have read and agreed to the published version of the manuscript.

Declaration of Competing Interest

We declare no conflict of interest.

Acknowledgments

The research was supported by the National Key R&D Program of China (2021YFD1801105), the National Natural Science Foundation of China (31872474), the Natural Science Foundation of Heilongjiang Province of China (YQ2020C023), and the Central Public-interest Scientific Institution Basal Research Fund (no.1610302020001).

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