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Original article

Effects of porcine epidemic diarrhea virus infection on Toll-like receptor expression and cytokine levels in porcine intestinal epithelial cells

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Abstract

To explore the role of Toll-like receptors (TLRs) and interferon (IFN) in the innate immunity against porcine epidemic diarrhea virus (PEDV), we detected the expression of TLR genes in PEDV-infected IPEC-J2 cells by real-time PCR. We also detected the level of interferon α (IFN-α) and interferon γ (IFN-γ) by enzyme-linked immunosorbent assay (ELISA). Results showed that IPEC-J2 cells exhibited a clear pathological change after PEDV infection at 24 h. In addition, *TLR7*, *TLR9* and *TLR10* expressions were significantly upregulated in PEDV-infected IPEC-J2 cells at 24 h. Interestingly, the expression patterns of *TLR2* and *TLR4* were consistent at different stages of PEDV infection. The expression level of *TLR3* decreased significantly with the increase of infection time, but the expression levels of *TLR5* and *TLR8* genes at 6 h and 12 h were significantly lower than those in the control group ($p<0.01$). There were significant correlations among the expression levels of *TLR* genes ($p<0.05$). Cytokine detection showed that the secretion level of *IFN-α* in the PEDV-infected group was significantly higher than that in the control group ($p<0.01$), and *IFN-γ* at 6 h and 12 h after PEDV infection was significantly higher than that in control group ($p<0.01$). Therefore, our results suggest that PEDV infection can induce innate immune responses in intestinal porcine jejunum epithelial cells, leading to changes in the expression of Toll-like receptors, and can regulate the resistance to virus infection by affecting the release levels of downstream cytokines.

Key words: pig, porcine epidemic diarrhea virus,TLRs, innate immunity

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Introduction

Toll-like receptors (TLRs) are fundamental sensor molecules of the host innate immune system, which detect conserved molecular signatures of a wide range of microbial pathogens and initiate innate immune responses via distinct signaling pathways. Various TLRs are implicated in the early interplay of host cells with invading viruses and regulates viral replication and host responses, ultimately impacting on viral pathogenesis (Wu et al. 2007). TLRs may be expressed extra- or intracellularly. Some TLRs (TLR 1, 2, 4, 5, 6 and 10) are expressed on the cell surface, and recognize components of microbial cell walls and membranes such as lipoproteins and peptidoglycans. Others (TLR 3, 7, 8, and 9) are found almost exclusively in intracellular compartments such as endosomes, and can detect distinct forms of viral nucleic acids and are critical for the recognition of viral genetic materials in end lysosomal compartments and for the initiation of antiviral responses (Akira et al. 2006). TLR2 and TLR4 have been implicated in the recognition of viral structural and nonstructural proteins leading to inflammatory cytokine production (Lester et al. 2014).

Porcine epidemic diarrhea (PED) is a viral infectious disease caused by porcine epidemic diarrhea virus (PEDV), which results in high mortality of suckling pigs and substantial economic losses in the pig industry. Clinically, pigs infected with PEDV have severe diarrhea and vomiting, leading to death by dehydration within a few days of infection (Li et al. 2016). PEDV belongs to the genus Alpha coronavirus in the family Coronaviridae. Epidemic PEDV strains are highly enteropathogenic and acutely infect villous epithelial cells of the entire small and large intestines, but the jejunum and ileum are the primary sites of infection. During the early stages of PEDV infection, necrosis and exfoliation of infected villous epithelial cells is pronounced, resulting in acute, severe villous atrophy, and disruption of the integrity of the intestinal barrier in vivo (Jung et al. 2015, Beall et al. 2016).

It has been shown that the innate immune system protects colon from dextran sulfate sodium (DSS)-induced intestinal epithelial injury by the ability of TLRs to recognize commensal bacterial products (Rakoff-Nahoum et al. 2004). In relation to the mechanism of action, it has been shown that connexins interact with commensal TLRs in intestinal epithelial cells to maintain their normal distribution, thereby preserving the epithelial barrier function (Cario et al. 2007).

Toll-like receptors can clear invading viruses by mediating innate immune responses and producing immune effector molecules. To survive the host innate defense mechanisms, many viruses have developed strategies to evade or counteract signaling through the TLR pathways, creating an advantageous environment for their propagation. Ten porcine TLR proteins (TLR1-TLR10) have been cloned and characterized (Dai et al. 2018). However, few studies have been conducted on the expression level changes of TLR genes and the effects on cytokine secretion levels after PEDV infection in IPEC-J2 cells. In this study, IPEC-J2 cells were infected with PEDV for different periods of time, and the expression levels of TLR genes at different times of infection were detected by real-time PCR to analyze the relationship between the expression levels of TLR genes and PEDV infection at the cellular level. The effect of PEDV infection time on *IFN-α* and *IFN-γ* cytokine secretion was also examined. This study provided a basis for the future study of swine TLR gene function and its regulation in PEDV virus infection.

Materials and Methods

Materials

African green monkey kidney cells (Vero) were derived from the China Center for Type Culture Collection, and intestinal porcine jejunum epithelial cells (IPEC-J2) were provided by the University of Pennsylvania, USA. PEDV CV777 was provided by the China Agricultural University. Fetal bovine serum (FBS) and Dulbecco's modified eagle medium (DMEM) were purchased from Gibco (NY, USA). TRIZOL reagent was purchased from Invitrogen (CA, USA), reverse transcription (Hi Script Q Select RT Super Mix for qPCR) and quantitative fluorescence (AceQ qPCR SYBR Green Master Mix) kits were both purchased from Vazyme Biotech Co., Ltd. (Nanjing, China). Porcine *IFN-α* and *IFN-γ* ELISA kits were purchased from AssayPro (MO, USA).

Primer design and sequence synthesis

Real-time PCR primers used for the *TLR1-10* gene were as previously reported by Uddin (Uddin et al. 2013). The *GAPDH* gene was used as an internal control, and primers were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). Primer information is shown in Table 1.

PEDV infection of IPEC-J2 cells

IPEC-J2 cells were inoculated to 24-well plates and incubated with DMEM culture medium containing 10% FBS in an incubator under the conditions of 37°C and 5% CO_2. When the coverage of cells reached ~80%,

Table 1. Primers used for real-time PCR.

Gene	Accession number	Length (bp)	Primer sequence 5'→3'
TLR1	NM_001031775	277	F: AGATTTCGTGCCACCCTATG
			R:CCTGGGGGATAAACAATGTG
TLR2	NM_213761	163	F:TGCTATGACGCTTTCGTGTC
			R:CGATGGAGTCGATGATGTTG
TLR3	NM_001097444	149	F:GAGCAGGAGTTTGCCTTGTC
			R:GGAGGTCATCGGGTATTTGA
TLR4	NM_001113039	234	F: TCATCCAGGAAGGTTTCCAC
			R: TGTCCTCCCACTCCAGGTAG
TLR5	NM_001123202	114	F: GGTCCCTGCCTCAGTATCAA
			R: TGTTGAGAAACCAGCTGACG
TLR6	NM_213760.1	170	F: TCAAGCATTTGGACCTCTCA
			R: TTCCAAATCCAGAAGGATGC
TLR7	NM_001097434	317	F: TCTGCCCTGTGATGTCAGTC
			R: GCTGGTTTCCATCCAGGTAA
TLR8	NM_214187	241	F: CTGGGATGCTTGGTTCATCT
			R: CATGAGGTTGTCGATGATGG
TLR9	NM_213958	205	F: AGGGAGACCTCTATCTCCGC
			R: AAGTCCAGGGTTTCCAGCTT
TLR10	NM_001030534	128	F: GCCCAAGGATAGGCGTAAAT
			R: CTCGAGACCCTTCATTCAGC
GAPDH	DQ178124	100	F: ACTCACTCTTCTACCTTTGATGCTG
			R: TGTTGCTGTAGCCAAATTCA

the culture medium was discarded and the cells were washed three times with PBS.

Control group: IPEC-J2 cells were incubated without virus. Virus infection group: 500 μL PEDV CV777 virus was added to each well. After 2 hours of culture, the unattached viruses were discarded. The cells were washed 3 times with PBS, and complete culture medium was replaced. Infected cells were cultured at 37°C in a 5% CO_2 incubator for different times (6, 12, 24 h), and total RNA was then extracted; each treatment had three repeats.

Cytokines detection of *IFN-α* and *IFN-γ*

The supernatants of the control group and the different time treatment groups were collected. The levels of two cytokines, interferon α (*IFN-α*) and interferon γ (*IFN-γ*) were measured using ELISA kits (AssayPro, USA). The assay procedure and standard curve generation were carried out according to the instructions provided with the kit.

Extraction of total RNA and real-time PCR

Total RNA was extracted using the Trizol Reagent (Takara, China), according to the manufacturer's instructions. RNA was reversed into cDNA and used as the template to detect and analyze the transcript levels of the TLRs. The house keeping gene *GAPDH* was used as an internal control to normalize the expression levels. We performed qPCR using the ABI 7500 system (Applied Biosystems, USA). The reaction conditions were 95°C for 15 s, followed by 40 cycles of 95°C for 5 s and 62°C for 34 s. The dissociation curve was analyzed after amplification. The reaction conditions were 95°C for 15 s, 62°C for 60 s, 95°C for 15 s, and 60°C for 15 s.

Statistical analysis

The $2^{-\Delta\Delta Ct}$ method was used to detect the transcript levels of the genes (Livak et al. 2001). The T- test, correlation analysis and one-way ANOVA method in SPSS

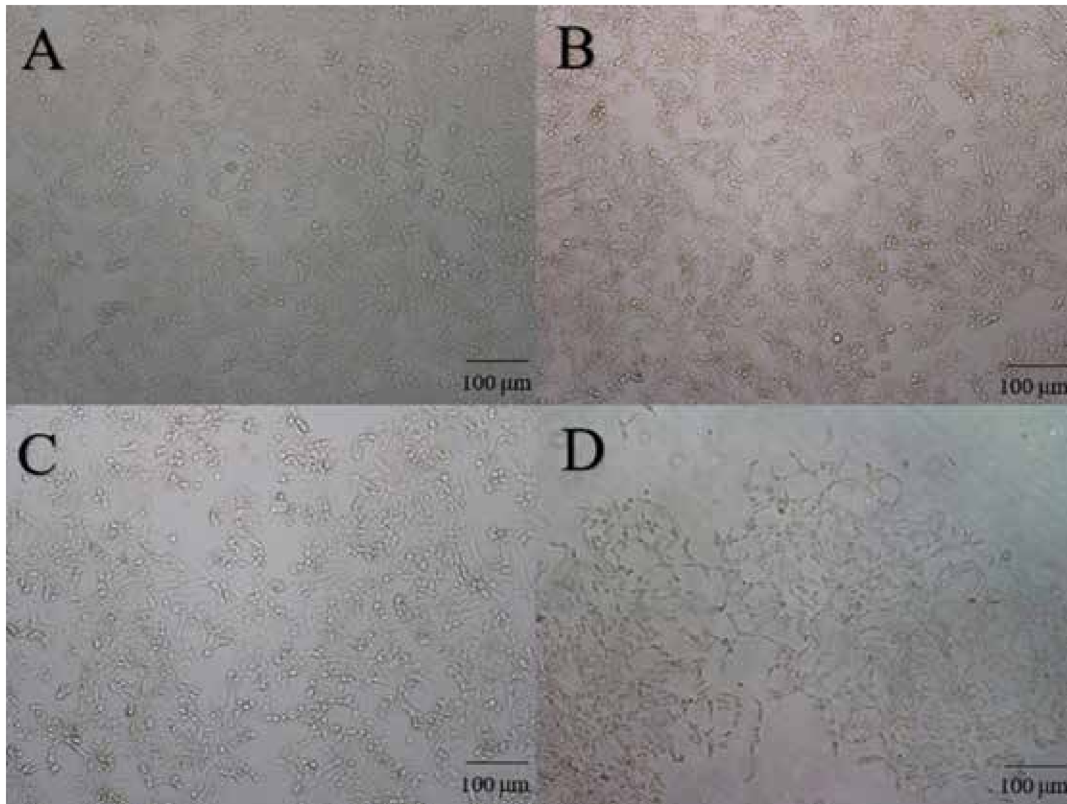


Fig. 1. Cell morphology after different infection times. A. IPEC-J2 cells incubated without virus. B. IPEC-J2 cells infected with PEDV for 6 h. C. IPEC-J2 cells infected with PEDV for 12 h. D. IPEC-J2 cells infected with PEDV for 24 h.

18 software were provided for difference analysis of transcript levels of genes and cytokine levels between the control group and virus infection group. Each independent experiment or treatment was performed in triplicate.

Results

Morphological changes of cells

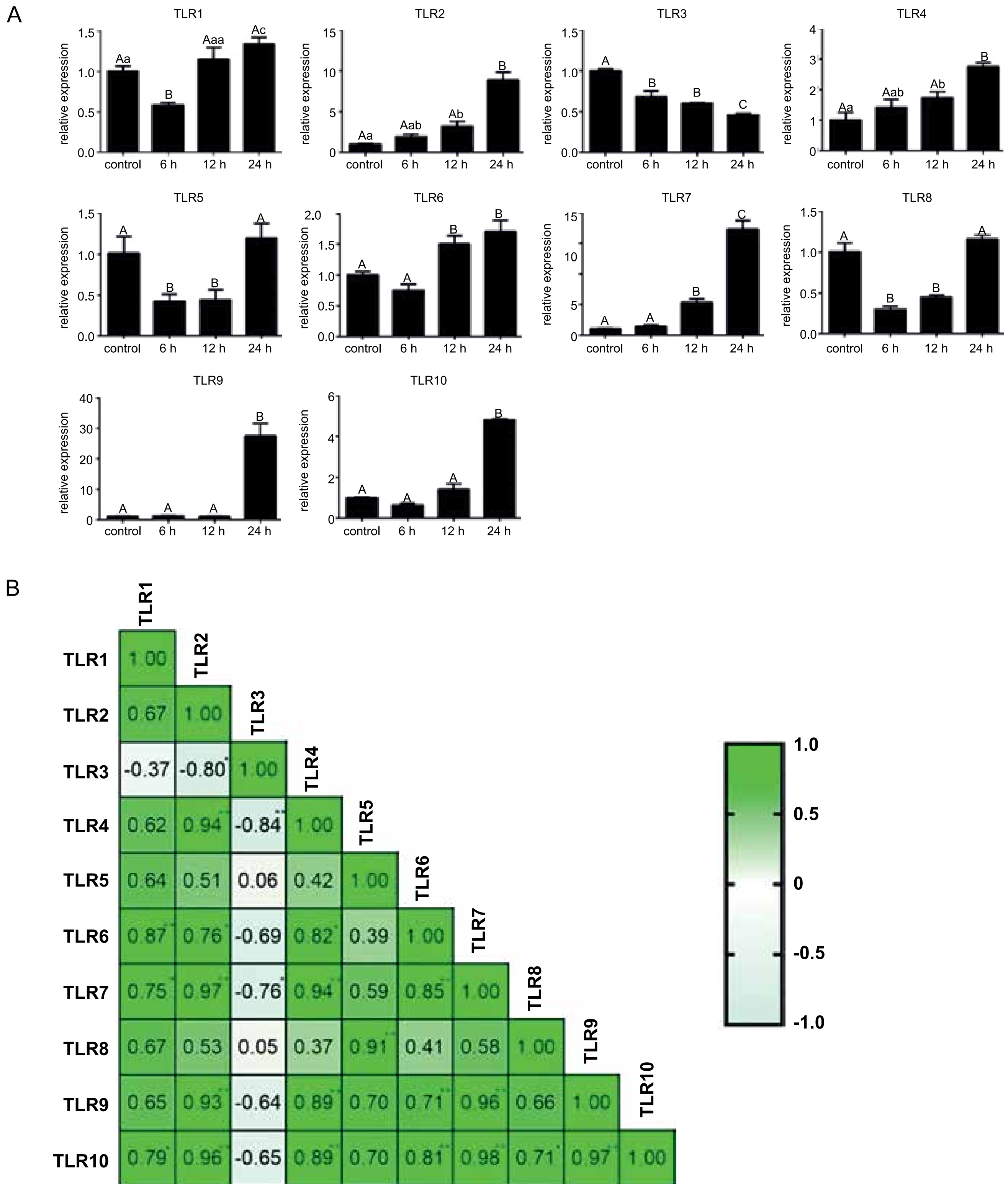
Compared to the IPEC-J2 without virus infection (Fig.1A), cells still maintained normal morphology at 6 h of virus infection (Fig.1B); cells had shrunk and had a slight change in morphology at 12 h of virus infection (Fig.1C); cells had developed significant lesions at 24 h of infection (Fig. 1D).

Influence of PEDV Infection on *TLR1-10* Gene in IPEC-J2 Cells

Using the gene expression level of the control group as a reference, we quantified the mRNA expression levels of the *TLR1-10* gene after virus treatment for different time points. As shown in Fig. 2A, the expression levels of *TLR2*, TLR4, *TLR7*, *TLR9* and *TLR10* genes at 24 h were significantly higher than those in the control group at 6 h and 12 h ($p<0.01$). The expression levels of *TLR5* and *TLR8* genes at 6 h and 12 h were significantly lower than those in the control group ($p<0.01$), and their expression levels at 24 h were significantly higher than those at 6 h and 12 h, but not significantly different from those in the control group ($p>0.05$). The expression level of *TLR3* decreased significantly with the increase of infection time, but the difference was not significant between 6 h and 12 h ($p>0.05$). The expression levels of *TLR1* and *TLR6* genes decreased at 6 h after virus infection, and then showed an upward trend. To explore the relationship between genes in the antiviral responses, correlation analysis was performed based on gene expression levels (Fig. 2B). The results showed a highly significant correlation between multiple genes such as *TLR1* and *TLR6*, and between *TLR2*, *TLR4*, *TLR7*, *TLR9* and *TLR10*.

Cytokine *IFN-α* and *IFN-γ* Secretion Levels after Virus Infection

ELISA kits were used to detect the secretion levels of cytokines *IFN-α* and *IFN-γ* (Fig.3). The results showed that the secretion level of *IFN-α* increased continuously during the course of PEDV infection, and the secretion level of *IFN-α* at different infection times was extremely significantly different from



	TLR1	TLR2	TLR3	TLR4	TLR5	TLR6	TLR7	TLR8	TLR9	TLR10
TLR1	1.00									
TLR2	0.67	1.00								
TLR3	-0.37	-0.80*	1.00							
TLR4	0.62	0.94**	-0.84**	1.00						
TLR5	0.64	0.51	0.06	0.42	1.00					
TLR6	0.87*	0.76*	-0.69	0.82*	0.39	1.00				
TLR7	0.75*	0.97**	-0.76*	0.94**	0.59	0.85**	1.00			
TLR8	0.67	0.53	0.05	0.37	0.91**	0.41	0.58	1.00		
TLR9	0.65	0.93**	-0.64	0.89**	0.70	0.71**	0.96**	0.66	1.00	
TLR10	0.79*	0.96**	-0.65	0.89**	0.70	0.81**	0.98**	0.71*	0.97**	1.00

Fig. 2. Gene expression and correlation coefficient. A. Expression levels of TLRs after PEDV infection for different periods**.** Different lowercase letters represent *P*<0.05, different uppercase letters represent *p*<0.01, and the same letters represent no significant difference. B. Correlation coefficient of fluorescence quantitative results of TLRs. Note: ** *p*<0.01, * *p*<0.05.

the control group (p<0.01). In the process of PEDV infection, the secretion level of *IFN-γ* in the virus infection group was higher than that in the control group and reached the maximum level at 6 h of infection. In addition, the secretion levels of *IFN-γ* at 6 h and 12 h of infection were significantly higher than that in the control group (p<0.01).

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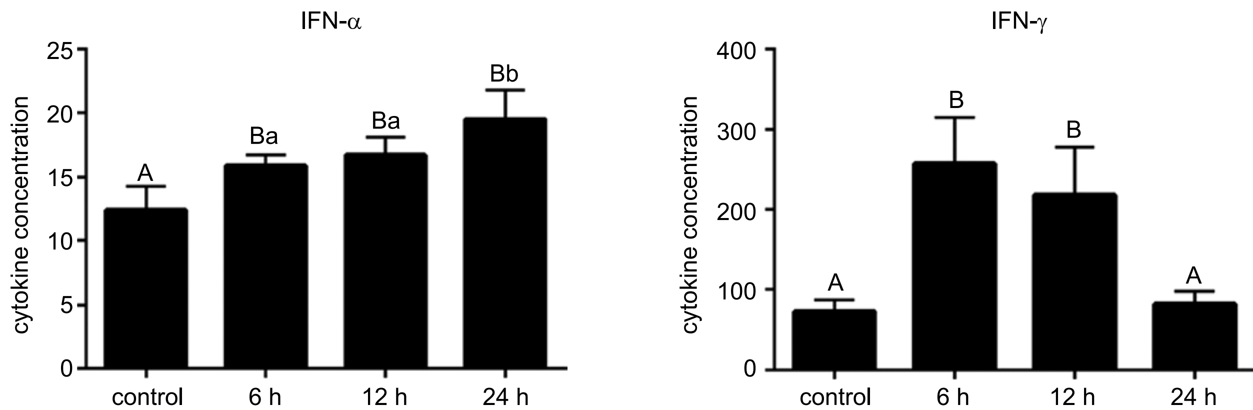

Fig. 3. *IFN-α* and *IFN-γ* secretion levels after PEDV infection. Different lowercase letters represent $p<0.05$, different uppercase letters represent $p<0.01$, and the same letters represent no significant difference.

Discussion

The host evokes innate immune responses to eliminate invading pathogens by detecting the presence of infection (Kawai et al. 2008). Each TLR detects distinct pathogen-associated molecular patterns (PAMPs) derived from viruses, bacteria, mycobacteria, fungi and parasites. Upon recognition of respective PAMPs, TLRs recruit a specific set of adaptor molecules that harbor a Toll/IL-1receptor-like domain, such as MyD88 and TIR domain-containing adapter inducing interferon-β, and initiate downstream signaling events that lead to the secretion of inflammatory cytokines (Medzhitov et al. 2002, Kawai et al. 2011). Some viruses utilize more than one TLR to activate their proinflammatory responses, and different TLR genes synergistically stimulate innate antiviral activities (Sørensen et al. 2008, So et al. 2009). In this study, the effects of PEDV infection on the expression of TLR genes and the secretion levels of some cytokines were analyzed. The expression patterns of *TLR2* and *TLR4* were consistent at different stages of PEDV infection. In the PEDV infection process, the expression levels of *TLR7*, *TLR9* and *TLR10* were significantly upregulated in the late stage of viral infection. In addition, the expression levels of *TLR3*, *TLR5* and *TLR8* showed a decreased trend. In the process of PEDV infection, the secretion levels of *IFN-α* and *IFN-γ* at different infection times were higher than those in the control group. The changes of TLR genes and the expression of important cytokines during PEDV infection were preliminarily investigated in order to provide a reference for further study on the role of TLR genes in PEDV infection.

TLR2 and *TLR4* mainly recognize viral envelope proteins or viral proteins released into the extracellular environment (Nazli et al. 2013). *TLR2* forms a heterodimer with *TLR1* or *TLR6* to function (Henrick et al. 2015). Oxidized low-density lipoprotein and amyloid-β trigger inflammatory signaling through a heterodimer of *TLR4* and *TLR6* (Wilkinson et al. 2010). In addition, it has been shownthat there is an interaction between *TLR2* and *TLR4*, which may mediate the production of cytokines by forming dimers and initiate the innate immune response to antiviral infection (Fernandez-Lizarbe et al. 2013, Perkins et al. 2013). In this study, *TLR2* and *TLR4* had a consistent expression pattern during PEDV infection, and the expression level was significantly correlated; however, the expression pattern between *TLR2* and *TLR1*, *TLR2* and *TLR6* after infection was not consistent, and the correlation between *TLR2* and *TLR1* expression level was not significant. It is speculated that *TLR2* and *TLR4* form a heterodimer in the process of PEDV infection of IPEC-J2 cells, and then recognize the virus to mediate immune response. However, this remains to be further experimentally validated.

It has been experimentally confirmed that IPEC-J2 cells are susceptible to PEDV (Zhao et al. 2014). Lesions of IPEC-J2 cells were also observed at 12 h of PEDV infection in this study, and significant lesions were observed at 24 h. *TLR7* is located intracellularly and mainly recognizes viruses that enter the nucleus by endocytosis (Diebold 2008). Through the endosomal vesicle acidification pathway, *TLR7* can identify single-stranded RNA sequences rich in GU and AU in RNA virus (Lund et al. 2004). After PEDV infection of IPEC-J2 cellsn for 12 h, the expression level of *TLR7* was significantly upregulated, which is consistent with the immune surveillance of *TLR7*. *TLR9* gene mediated antiviral innate immunity recognizes double-stranded DNA viruses such as poxviruses and some RNA viruses (Tabeta et al. 2004, Lai et al. 2018). Cao et al. (2015) found that PEDV infection could activate NF-κB through the *TLR9* signaling pathway. Borca et al. (2008) carried out a study of porcine macrophages infected with the classical swine fever virus (CSFV) Brescia strain and found that the expression of the *TLR10* gene was significantly altered, and it was speculated that

TLR10 plays a role in the antiviral response elicited by CSFV. PEDV infection can cause morphological changes of cells, and induce the significant upregulation of *TLR7*, *TLR9* and *TLR10* mRNA transcription levels in the cells. *TLR7*, *TLR9* and *TLR10* may recognize PEDV; they are activated by certain ligands and exert antiviral effects after viral infection.

TLR3 recognizes dsRNA viruses and the dsRNA produced during replication of ssRNA virus and dsDNA viruses, and coronaviruses can escape the host innate immune response by blocking TLR3-mediated signaling pathways (Kawai et al. 2011, Guo et al. 2016). *TLR8* recognizes single-stranded RNA viruses, such as the human immunodeficiency virus, which mainly causes the activation of NF-κB and promotes the expression of inflammatory cytokines (Liu et al. 2005). Huang et al. (2017) infected SPF chickens with avian reovirus and detected a significant change in the expression level of *TLR5* in the joints, suggesting that *TLR5* may be involved in the immunity to avian reovirus. The present study showed that the expression of *TLR5* and *TLR8* genes was significantly decreased during PEDV infection of IPEC-J2 cells, and it is speculated that PEDV may inhibit the recognition and response of innate immunity by downregulating the expression of *TLR5* or *TLR8*.

IFN-α is a type I interferon and a key effector molecule in the antiviral innate immune response. In the early stages of viral infection, the host defense ability competes with the virus infection ability and, in this process, innate immunity plays an important role in limiting the early infection and transmission of the virus (Yang et al. 2004). In the process of PEDV infection of IPEC-J2 cells, the secretion levels of *IFN-α* at different stages of infection were higher than those in the controlgroup, indicating that the cells exert antiviral effects by activating the transcription of type I IFN genes. *IFN-γ* is the only type II interferon, also known as immune interferon. The production of *IFN-γ* depends on the regulation of other cytokines, and *IFN-α/*β negatively regulate it (Nguyen et al. 2000). Some studies have shown that porcine *IFN-γ* can significantly inhibit the proliferation of porcine reproductive and respiratory syndrome virus and is also involved in cellular immunity against CSFV (Cao et al. 2004). According to the test results, the secretion level of *IFN-γ* in the virus-infected group was higher than that in the control group, indicating that it played a role in the anti-PEDV process in IPEC-J2 cells. The decrease of secretion level in the late stage of infection may be derived from the inhibitory effect of high levels of *IFN-α*.

In this study, PEDV was used to infect intestinal porcine jejunum epithelial cells. The antiviral effects of TLRs were analyzed by measuring their expression changes in the cells before and after stimulation. It was confirmed at the cellular level that PEDV infection could induce an innate immune response in intestinal porcine jejunum epithelial cells, leading to expression changes of Toll-like receptors and downstream cytokines. In this study, we preliminarily revealed the expression regulation of Toll-like receptor family genes in intestinal porcine jejunum epithelial cells infected with PEDV, providing a theoretical reference and experimental basis for further study on the molecular mechanism of TLR genes regulating porcine epidemic diarrhea.

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