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Differential gene modulation of pattern-recognition receptor TLR and RIG-I-like and downstream mediators on intestinal mucosa of pigs infected with PEDV non S-INDEL and PEDV S-INDEL strains

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ABSTRACT

Porcine epidemic diarrhea virus (PEDV) strains can be divided into non-S-INDEL and S-INDEL strains. PEDV pathogenesis is strain-specific, and studies in neonatal pigs have demonstrated that the PEDV non-S-INDEL strains are more pathogenic than the PEDV S-INDEL strains. RNA viruses, including PEDV, can interact with a large number of pattern recognition receptors (PRRs) in the intestinal mucosa, including toll-like receptors (TLRs) and RIG-I-like receptors (RLRs). We investigated the differential gene modulation of TLRs, RIG-I, and downstream mediators on the intestinal mucosa of neonatal pigs infected with PEDV S-INDEL and non-S-INDEL strains. Ten five-day-old piglets were inoculated orally with 10 ml of 10^4 $TCDI_{50}$/ml of either PEDV non-S-INDEL or S-INDEL strains. PEDV S-INDEL infection induced pro-inflammatory cytokines through the non-canonical NF-κB signaling pathway by activating RIG-I. In contrast, PEDV non-S-INDEL infection suppressed the induction of pro-inflammatory cytokines and type 1 interferon production by down-regulation of TLRs and downstream signaling molecules.

1. Introduction

Porcine epidemic diarrhea virus (PEDV) belongs to the order *Nidovirales,* family *Coronaviridae*, and genus *Alphacoronavirus,* and is an enveloped virus with a single-stranded positive RNA genome. It contains four structural proteins-spike (S), membrane (M), nucleocapsid (N), and envelop (*E*)-responsible for viral infectivity and the induction of immune response (Duarte and Laude, 1994; Duarte et al., 1994; Kocherhans et al., 2001). PEDV causes enteric disease, resulting in significant morbidity and mortality in neonatal pigs, and has been reported as a major source of substantial economic losses in most swine producer countries (J. Chen et al., 2010; Q. Chen et al., 2013; X. Chen et al., 2012; Cima, 2014; Song and Park, 2012; Takahashi et al., 1983). In 2014, a less pathogenic PEDV strain was reported in the United States and several other swine producer countries (Chen et al., 2016; Vlasova et al., 2014; Yamamoto et al., 2015). This new strain presented insertions and deletions on the amino terminal region of the S protein. On the basis of differences in the S gene and virulence, emerging PEDV strains can be divided into non-S-INDEL (S gene insertions and deletions) and S-INDEL strains (Vlasova et al., 2014). PEDV pathogenesis is strain-specific, and pathogenesis studies in neonatal pigs have demonstrated that the PEDV non-S-INDEL strain is more pathogenic than the PEDV S-INDEL strain (Chen et al., 2016; Wang et al., 2016; Yamamoto et al., 2015). PEDV pathogenesis is also inversely correlated with the age of the animals. In adult pigs, PEDV infection is self-resolving regardless of previous PEDV immune status. Moreover, PEDV S-INDEL was shown to be clinically relevant in neonates, but clinical disease could not be reproduced in pigs older than three weeks (Annamalai et al., 2015; Chen et al., 2016).

Host pattern recognition receptors (PRRs), such as toll-like receptors (TLRs) and the cytosolic retinoic acid-inducible gene-1 (RIG-1)-like receptors, recognize pathogen-associated molecular patterns (PAMPs) during viral infection (Akira et al., 2006; Alexopoulou et al., 2001b; Takeuchi and Akira, 2010; Uematsu and Akira, 2008). This interaction triggers the interferon regulatory transcription factor (IRF3/7) and activates NF-κB, which modulates the expression of several pro-inflammatory cytokines and chemokines. Type 1 interferon alpha/beta (IFNα/IFNβ) are the two essential cytokines that can control viral infections (Kawai et al., 2005; Seth, 2005; Xu et al., 2005). Within nidoviruses, acute respiratory syndrome coronavirus (SARS-CoV) and Middle-East respiratory syndrome coronavirus (MERS-CoV) can evade the host immune system by interfering with the NF-κB signaling

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pathway (DeDiego et al., 2014; Matthews et al., 2014), and the infectious bronchitis virus (IBV) inhibits the phosphorylation of kinases that are necessary to activate downstream signaling cascades (Chen et al., 2013; Devaraj et al., 2007; Kint et al., 2015a; Sun et al., 2012; Zhou, 2007).

Two signaling pathways, known as the classical (canonical) pathway and the alternative (non-canonical) pathway (Kawai and Akira, 2010; Loo and Gale, 2011), lead to the activation of NF-κB. The canonical pathway includes the recruitment of the myeloid differentiation primary response gene 88 (MyD88), containing the toll/interleukin-1 receptor (TIR) domain for eventual activation of NF-κB and induction of type I interferons (Kawai and Akira, 2007, 2010; Thompson and Locarnini, 2007). In addition, endosomal receptor TLR3 contains exclusively TIR-domain-containing adapter-inducing interferon-β (TRIF) adapter proteins that interact with TRAF6, which induces IRF3/7 similar to the MyD88 pathway (Yamamoto et al., 2003; Zhengfan, 2004;). Finally, both the MyD88 and TRIF pathways activate NF-κB and induce expression of the antiviral type I interferons (Thompson and Locarnini, 2007). The non-canonical pathway is TLR-independent, and NF-κB can be modulated by RIG-I-TRAF3 mediated through IRF3 (Devaraj et al., 2007).

Several *in vitro* studies have tried to elucidate the role of PEDV in innate immune response at the cellular level. It has been demonstrated that the nucleocapsid (N) protein of PEDV, during infection of HEK-293T cells, inhibited IFN-β production by annexing the vital interaction between IRF3 and TBK1 (Ding et al., 2014b). Other *in vitro* studies in porcine intestinal epithelial cells (IECs) determined that PEDV infection impeded the production of IFN-β by inhibiting the RIG-I pathway and hampering the activation of IRF3 (Cao et al., 2015a). Studies in Vero cells showed that PEDV infection degraded STAT-1 and disrupted the IFN response (Li et al., 2016). Hence, it has been shown that PEDV can regulate different immunological pathways *in vitro*; the main regulatory effect of PEDV on mucosal innate immunity and its strain-dependence on viral pathogenicity is still unknown.

The aim of this study was to investigate the differential gene modulation of pattern recognition TLR and RIG-I-like receptors and downstream mediators on the intestinal mucosa of neonatal pigs infected with PEDV non-S-INDEL and PEDV S-INDEL strains.

2. Materials and methods

2.1. Animal study

Thirty 5-days-old conventional piglets were selected for the molecular evaluation of the gene modulation of pattern recognition TLR and RIG-I-like receptors and downstream mediators on intestinal mucosa. The experimental design as well as information of the virus strains used in this study, kinetic of virus shedding, virus distribution in tissues, and pathogenicity was previously described in a PEDV clinical pathogenesis study (Chen et al., 2016). In brief, pigs were injected intramuscularly with a dose of Excede (Zoetis, Kalamazoo, MI) at time of delivery to Iowa State University, Laboratory Animal Resources facilities (Ames, IA). All pigs were confirmed negative for PEDV, PDCoV, TGEV, and porcine rotaviruses (groups A, B, and C) by virus-specific PCRs on rectal swabs, and seronegatives by PEDV indirect immunofluorescent assay. After one day of acclimation, six-day-old pigs were inoculated orogastrically with 10 ml of 10^4 $TCID_{50}$/ ml of PEDV non-S-INDEL (USA/IN19338/2013) or PEDV S-INDEL (USA/IL20697/2014), or 10 ml of virus-negative culture medium. Five pigs from each group were euthanized at three and seven days post-inoculation (dpi), respectively. Sections of distal small intestine of approximately 0.5 cm in length were snap frozen and saved at −80 °C. Frozen intestine tissue samples were aliquoted, placed in an RNAlater™ Stabilization Solution (Life Technologies, Carlsbad, CA), and kept at −80 °C until further use.

2.2. RNA extraction from porcine intestine tissue

RNA was extracted from 5 mg of porcine intestinal tissue using the Ambion® MagMAX™ total RNA isolation kit (Life Technologies) and a Kingfisher® 96 magnetic particle processor (Thermo Fisher Scientific, Waltham, MA) following the manufacturer's instructions. RNA was eluted into 70 μL of elution buffer and stored at −80 °C.

2.3. Expression of mRNA for TLRs, inflammatory signaling pathways, and cytokines on porcine intestinal mucosa

The relative quantification of gene expression of toll-like receptors TLR2, TLR3, TLR4, TLR7, TLR8, and TLR9 was evaluated on total RNA extracted from porcine intestinal tissues. Gene expression of inflammatory signaling pathway molecules, including RIG-1, TRIF, MyD88A, MyD88B, IRF7, TRAF6, NF-κB1 (p105) NF-κB1 (p50), and RelA (p65), was also conducted by SYBR-green RT- PCR on the RNA extracted from intestinal pig mucosa. Gene expression of cytokines IFN-α, IL-6, IL-12, and TNF-α in intestinal mucosa was also investigated following the same protocol described herein for the rest of the genes. All reactions were performed in triplicate and the relative gene expression of each target gene was evaluated in reference to the expression of housekeeping genes GAPDH and beta-actin. All primers were custom-synthesized (Integrated DNA Technologies, Inc., Coralville, IA) to target amplicons, with sizes ranging between 95 and 120 nt base pairs according to the cDNA sequence of each target gene, collected from the National Center for Biotechnology Information (NCBI) database (Table 1).

The mRNA expression levels were quantified according to the ΔΔCt method (Livak and Schmittgen, 2001). Briefly, the difference in cycle times, ΔCt, was determined as the difference between the tested gene and the reference housekeeping genes. The ΔΔCt was obtained by finding the difference between groups. The fold change was calculated as $2^{-\Delta\Delta Ct}$. Real-time RT-PCR was performed with eluted RNA and primers, mixed with the commercial Power SYBR Green RNA-to-C_T™ 1-Step Kit (Applied Biosystems, Foster City, CA), following the manufacturer's recommendations. The reverse transcription reaction was conducted at standard mode for 30 min at 48 °C, enzyme activation was conducted at 95 °C for 10 min using an Applied Biosystems™ 7500 real-time PCR instrument. The strands were denatured at 95 °C for 15 s, then annealed and extended at 60 °C for 1 min (40 cycles). A dissociation curve was obtained for each quantitative PCR run to assess its target specificity. The real-time RT-PCR was analyzed with a threshold fixed at a 0.1 setting. Cycle threshold (CT) values ≤ 35 were considered positive for the housekeeping and TLR gene expressions. All samples were tested in triplicate and the results were expressed as fold changes relative to the control animals.

2.4. Statistical analysis

Data were analyzed for normality by Kolmogorov-Smirnov test. The statistical significance between the two treatment groups was determined by non-parametric statistical analysis using the Mann-Whitney test. Significance was assessed at $p < 0.05$. Data analysis was performed using GraphPad Prism® (GraphPad Software Inc., La Jolla, CA).

3. Results

3.1. PEDV non S-INDEL and S-INDEL infection showed differential modulation on PRRs genes in intestinal mucosa

RNA viruses, including PEDV, can interact with a large number of pattern recognition receptors (PRRs) in the intestinal mucosa, such us toll-like receptors (TLRs) and RIG-I-like receptors (RLRs). This interaction plays a critical role in the activation of the innate immune