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# Age-dependent variation in innate immune responses to porcine epidemic diarrhea virus infection in suckling versus weaned pigs

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ABSTRACT

Porcine epidemic diarrhea (PED) is an enteric coronaviral infection that causes severe morbidity and mortality in suckling pigs, but less severe disease in older pigs. Consequently, it causes significant economic losses to the pork industry. There are limited studies on the innate immune responses to PED virus (PEDV) in pigs. The aims of our study were to investigate differences in innate immune responses to PEDV infection in suckling and weaned pigs and to examine if disease severity coincides with reduced innate immune responses. Weaned 26-day-old pigs ($n=20$) and 9-day-old nursing pigs ($n=20$) were assigned to PEDV inoculated or uninoculated control groups. The pigs were observed daily for clinical signs, virus shedding and were euthanized at post-inoculation days (PIDs) 1 and 5 to assay immune responses. Blood samples were collected at PIDs 1, 3 and 5. The natural killer (NK) cell frequencies, NK cell activities (lysis of target K562 tumor cells in vitro), CD3+CD4+ T cell and CD3+CD8+ T cell frequencies were measured in blood and ileum at PIDs 1 and 5. The PEDV infected suckling pigs showed severe diarrhea and vomiting at PID 1, whereas the PEDV infected weaned pigs showed milder clinical signs starting at PID 3. PEDV infected suckling pigs had significantly higher diarrhea scores, earlier fecal PEDV RNA shedding and significantly higher viremia (viral RNA in serum) compared to weaned pigs. There was no mortality in either infected suckling or infected weaned pigs. The control pigs not inoculated with PEDV did not show any clinical signs and no detectable fecal or serum PEDV RNA. Strikingly, PEDV infected suckling pigs had significantly lower NK cell frequencies, undetectable NK cell activity and lower IFNγ producing NK cells in blood and ileum compared to PEDV infected weaned pigs. Pro-inflammatory cytokine profiles of PEDV infected suckling pigs differed from those of PEDV infected weaned pigs and coincided with onset of fecal PEDV RNA shedding and serum PEDV RNA titers. The infected suckling pigs have higher and earlier increases in serum IFNα, but lower serum IL-8 and TNFα levels compared to infected weaned pigs. CD3+CD4+ T cell frequencies were significantly higher in ileum of suckling pigs than in weaned pigs, whereas there was no difference in CD3+CD8+ T cell frequencies. In conclusion, the observations of impaired lytic activity and IFN-γ production by NK cells in suckling pigs coincided with the increased severity of PEDV infection in the suckling pigs compared with the weaned pigs.



## 1. Introduction

Porcine epidemic diarrhea virus (PEDV) is an enteric coronavirus (genus *Alphacoronavirus*, family *Coronaviridae*, order *Nidovirales*) causing significant morbidity and mortality in suckling pigs. PEDV was first diagnosed in the USA in May, 2013 (Stevenson et al., 2013) and has spread throughout the USA and was also reported in Mexico and Canada (Vlasova et al., 2014). The estimated annual economic losses in the US from PEDV is $900 million to $1.8 billion (Paarlberg, 2014). PEDV causes severe enteric disease in suckling pigs (Chen et al., 2014; Stevenson et al., 2013), but milder disease in older weaned pigs (Madson et al., 2014). This is similar to earlier observations for another enteric coronavirus infection of pigs, transmissible gastroenteritis (TGE) (Saif et al., 1994). Therefore, similar to TGE virus (TGEV) infection, biofeedback of intestinal contents of affected pigs to older pigs to build herd immunity is considered as an important method to reduce losses from PEDV (Jung and Saif, 2015).

Viral infections induce both innate and adaptive immunity. Innate immunity involves production of cytokines and interferons as well as recruitment of innate immune cells such as

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dendritic cells, macrophages and natural killer (NK) cells (Rouse and Sehrawat, 2010). The innate immune response plays a significant role in controlling primary viral infections and in development of adaptive immune responses (Aoshi et al., 2011; Janeway and Medzhitov, 2002). NK cells are innate immune cells that display cytotoxic action against virus infected host cells and tumor cells (Campbell and Hasegawa, 2013; Herberman et al., 1975; Trinchieri, 1989) and thus play an important initial role in containing the viral infection. NK cells are also a major source of certain cytokines such as IFNγ and TNFα (Fauriat et al., 2010; Vivier et al., 2008). They play a key role in initial clearance of infection in viral diseases (Brandstadter and Yang, 2011).

Cytokines are important in viral infections in that they are necessary for cell to cell communication for inflammation and immune responses (Akira and Kishimoto, 1992). The early cytokines secreted during a viral infection help to modulate immune responses. The cytokines examined in this study are early cytokines that have mainly proinflammatory and antiviral action. Interferons are a group of cytokines whose major function is antiviral activity (Isaacs and Lindenmann, 1957; Wheelock, 1965). IFNα is a type I interferon produced by most cells in response to viral infection, with the major source being innate immune cells such as monocytes and dendritic cells (Trinchieri et al., 1978). IFNγ is a type II interferon produced initially by innate immune cells such as macrophages, dendritic cells and NK cells, and later on by activated T cells (Sen, 2001). IFNγ is important in enhancing the activities of phagocytic cells such as macrophages and NK cells (Carnaud et al., 1999). IL-8 is produced by various cell types and is a proinflammatory cytokine due to its chemoattractive properties for inflammatory cells (Arndt et al., 1996; Huber et al., 1991). IL-17 is a proinflammatory cytokine secreted by Th17 cells, as well as γδT cells (innate immune cells in mucosa) (Jin and Dong, 2013). It stimulates the inflammatory response to viral infections (Ryzhakov et al., 2011). IL-12 is a proinflammatory cytokine produced mainly by phagocytic cells and is involved in activation of NK cell activity including IFNγ production by NK cells (Trinchieri, 1995). TNFα is a proinflammatory cytokine secreted mainly by macrophages that regulates cell death, differentiation and inflammation (Bradley, 2008). Studies of rotavirus infection of children showed that the cytokine responses varied depending on severity of clinical signs in individuals (Jiang et al., 2003). Studies of human rotavirus infected gnotobiotic pigs showed similarly that the proinflammatory cytokine responses were more marked with virulent virus compared with attenuated virus infection (Azevedo et al., 2006). The above cytokines were examined in the present study to understand if differences in proinflammatory cytokine responses between suckling and weaned pigs may be involved in susceptibility of suckling pigs to severe disease by PEDV infection.

There is a lack of information on innate immune responses of young and older pigs to PEDV infection that might explain some of the differences in disease severity between young and older pigs. In the present study, we investigated the innate immune responses such as cytokine and NK cell activity as well as changes in frequencies of T cells to examine if differences coincide with the higher disease severity of suckling versus weaned pigs.

## 2. Materials and methods

### 2.1. Virus

The virus inoculum used in this study was the wild-type virulent US PEDV strain PC21A which was from the intestinal contents of a PEDV positive field piglet, then serially passaged two times in gnotobiotic pigs (Jung et al., 2014). The original sample was negative by PCR for other enteric viruses such as TGEV/porcine respiratory coronavirus (PRCV), porcine deltacoronavirus, rotavirus groups A, B, and C, porcine enteric caliciviruses, St-Valerien-like viruses, porcine astroviruses, enterovirus, kobuvirus, and bocavirus (Amimo et al., 2013a,b; Chung et al., 2005; Jung et al., 2015b; Kim et al., 2000; Sisay et al., 2013; Wang et al., 2011). Immune electron microscopy of the original sample using gnotobiotic pig hyperimmune serum to PEDV showed only PEDV particles. The gnotobiotic pig passaged PC21A intestinal contents were diluted in minimum essential medium (MEM) and used as inoculum in this study as noted below.

### 2.2. Experimental pigs and infection

Seronegative pregnant sows and 26-day-old, PEDV-seronegative weaned, Large White × Duroc crossbred pigs were obtained from a PEDV-free specific pathogen free (SPF) (confirmed by history, lack of qRT-PCR-PEDV positive fecal samples and PEDV antibodies) swine herd of The Ohio State University. The SPF OSU herd was also seronegative for antibodies to porcine respiratory and reproductive syndrome virus, PRCV, TGEV and porcine circovirus type 2. The sows farrowed naturally and nursed their piglets until the end of the study. The four experimental groups in the study were as follows. Group 1: PEDV inoculated 9-day-old suckling pigs ($n=9$); Group 2: MEM only inoculated 9-day-old suckling pigs ($n=11$); Group 3: PEDV inoculated 26-day-old weaned pigs ($n=11$); Group 4: MEM only inoculated 26-day-old weaned pigs ($n=9$). All experimental procedures on animals were approved by the Institutional Animal Care and Use Committee of The Ohio State University. Pigs in PEDV groups were inoculated orally with PEDV inoculum [8.9 $\log_{10}$ GE (genomic equivalents) (≈2.9 $\log_{10}$ plaque forming unit)/pig] and pigs in MEM only inoculated groups received MEM. The inoculation dose was based on a previous pathogenicity study in our lab (Jung et al., 2014). Following PEDV inoculation, pigs were monitored for clinical signs daily until necropsy. Diarrhea was assessed by scoring fecal consistency. Fecal consistency was scored as, 0 = solid; 1 = pasty; 2 = semi-liquid; 3 = liquid, with scores of 2 or more considered diarrheic. Inoculated and mock pigs ($n=3$-4/group at each time-point) were euthanized for immunological studies at an acute stage on post inoculation day (PID) 1 and at a later stage (PID 5) of infection. Blood samples were taken at PID 1 ($n=6$-8 pigs per group), PID 3 ($n=4$-8 pigs per group) and PID 5 (from euthanized pigs, $n=3$-4 per group) and separate serum aliquots were prepared for cytokine analysis and viral RNA quantification.

### 2.3. Analysis of PEDV RNA titers in fecal and serum samples

Rectal swabs were collected from all pigs on the designated PIDs to determine fecal PED viral shedding (PEDV RNA quantified by RT-qPCR). Two rectal swabs were suspended in 4 ml MEM (Jung et al., 2014). The RNA was extracted from 50 μl of serum or supernatants following centrifugation of the fecal suspensions (2000 × $g$ for 30 min at 4 °C), using the Mag-MAX Viral RNA Isolation Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. PEDV RNA titers in rectal swab supernatants and sera were determined by RT-qPCR as described previously (Jung et al., 2014).

### 2.4. Isolation of mononuclear cells (MNCs) from blood and ileum

Blood and ileum were collected on the day of euthanasia and processed for isolation of MNC as previously described (Yuan et al., 1996). The isolated cells were resuspended in RPMI medium (Roswell Park Memorial Institute medium) containing 8% fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM