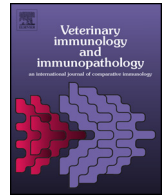Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

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

Thavamathi Annamalai, Linda J. Saif[*], Zhongyan Lu, Kwonil Jung[*]

Food Animal Health Research Program, Ohio Agricultural Research and Development Center, Department of Veterinary Preventive Medicine, The Ohio State University, Wooster, OH, USA

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.

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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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* Corresponding authors at: Food Animal Health Research Program, Ohio Agricultural Research and Development Center, College of Food, Agriculture and Environmental Sciences, Department of Veterinary Preventive Medicine, The Ohio State University, 1680 Madison Ave., Wooster, OH 44691, USA.

E-mail addresses: saif.2@osu.edu (L.J. Saif), jung.221@osu.edu (K. Jung).

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 $\gamma\delta$T 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 $\times$ 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) ($\approx$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 $\times$ 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

Table 1
Fecal consistency scores, fecal and serum PEDV RNA in PEDV inoculated suckling piglets vs weaned piglets.

PID	Fecal virus RNA shedding (log$_{10}$ GE/ml in rectal swab fluid)						Fecal scores					
	Mean virus RNA shedding titers (SEM)		% virus RNA shedding		Viral RNA titers in serum (log$_{10}$ GE/ml) (SEM)		Mean fecal scores (SEM)		% with diarrhea		Mean cumulative fecal scores of PIDs 1, 3 and 5 (SEM)	
	Suckling pigs	Weaned pigs	Suckling pigs	Weaned pigs	Suckling pigs	Weaned pigs	Suckling pigs	Weaned pigs	Suckling pigs	Weaned pigs	Suckling pigs	Weaned pigs
1	11.9[a] (0.1)	4.9[b] (0.1)	100	12.5	7.7[a] (0.2)	3.8[b] (0)	3[a] (0)	0[b] (0.1)	100	0	3[a] (0)	0.7[b] (0.15)
3	9.2 (0.7)	11.0 (0.6)	100	100	6.4[a] (0.2)	5.1[b] (0.3)	3[a] (0)	1.1[b] (0.3)	100	37.5		
5	9.3[a] (0.4)	12.2[b] (0.4)	100	100	6.1[a] (0.2)	4.8[b] (0.4)	3[a] (0)	1.1[b] (0.1)	100	0		

Suckling pigs had more severe clinical signs, earlier fecal PEDV RNA shedding and higher serum PEDV RNA compared to weaned piglets. Following PEDV inoculation, pigs were monitored every day and rectal swabs and blood for serum were collected at PID 1, 3 and 5. Fecal consistency scores of 2 or more considered diarrheic. Fecal shedding and serum PEDV RNA were determined by RTqPCR. The detection limit of RT-qPCR was 10 genomic equivalents (GE) per reaction, corresponding to 4.8 log$_{10}$ GE/mL of rectal swab fluid or 3.8 GE/ml of serum. Therefore, viral RNA titers more than 4.8 log$_{10}$ GE/ml in rectal swab fluid or 3.8 GE/ml in serum are considered positive. Statistical analysis was done to compare suckling and weaned pigs (across rows) for different parameters measured. Values with different alphabetical superscript within a parameter and time point are considered different at $P < 0.05$. The uninoculated pigs did not show any clinical signs. The rectal swab fluids and serum of uninoculated pigs were tested at similar time points and no PEDV RNA was detected.

nonessential amino acids, 20 mM HEPES and antibiotics (E-RPMI) and used for assays.

2.5. NK cell assay

K562 (human erythroleukemia cell line) tumor cells were used as target cells and the assay was done as described previously with a few modifications (Cao et al., 2013; Park et al., 2013). The K562 cells were initially stained with Carboxy fluorescein succinimidyl ester (CFSE) (eBioscience, USA), washed and used for the assay. MNCs from blood and ileum were used as effector cells. Effector:target cell ratios of 25:1, 12.5:1 and 6.25:1 were used. The cells were mixed at the specified ratios and incubated overnight in E-RPMI at 37 °C. The cells were then incubated with 7-Aminoactinomycin D (7-AAD) (Life Technologies, USA) for 15 min at 4 °C to stain dead cells. The cells were examined by flow cytometry and the percentage of CFSE positive cells that were also stained with 7-AAD were assessed as dead K562 cells. CFSE labeled K562 cells incubated without MNCs and stained similarly with 7-AAD were used as controls for spontaneous death of K562 cells.

2.6. IFN-γ-producing CD3-CD4-CD8+ NK cells

The procedure was followed as described previously (Chattha et al., 2013; Yuan et al., 2008) with a few modifications. Mononuclear cells from blood and ileum were cultured for 18 h at 37 °C in E-RPMI. The protein transport inhibitor, Brefeldin A (10 mg/ml; Sigma–Aldrich, USA), was added for the last 5 h to prevent secretion of IFNγ produced by the cells. The cells were stained with CD3-FITC (fluorescein isothiocyanate) (clone PPT3; Southern Biotech, Birmingham, AL, USA), CD8-SPRD (spectral red) (clone 76-2-11; BD Biosciences, USA), and CD4-biotin followed by streptavidin APC (allophycocyanin) (BD Biosciences, USA) as secondary antibody. Samples were stained intracellularly with anti-porcine IFN-γ–PE (phycoerythrin) (clone P2G10; BD Biosciences, USA). CD3-CD4-CD8+ IFN-γ+ cells were expressed as percentage of CD3-CD4-CD8+ NK cells. Isotype antibody-labeled cells were used as controls.

2.7. T cell and NK cell frequencies

To determine the frequencies of T helper cells (CD3+CD4+), cytotoxic T cells (CD3+CD8+) and NK cells (CD3-CD4-CD8+), cell samples were stained with anti-porcine CD3-FITC, CD4-PE (clone 74-12-4; BD Biosciences), and CD8-SPRD for 15 min at 4 °C. The frequencies of T cells or NK cells were expressed as percentage of lymphocytes expressing the respective markers. Cells stained with isotype antibodies were used as controls.

2.8. Cytokine assays

Serum was separated by centrifuging blood at 1850 × g for 15 min, and the collected serum was stored at −20 °C until tested. IL-12, IL-8, IL-17 and IFNα were measured as previously described (Azevedo et al., 2006; Chattha et al., 2013). For TNFα, a porcine TNFα ELISA Kit was used per manufacturer's recommendations (Kingfisher Biotechnologies, St. Paul, MN).

2.9. Statistical analysis

All values are expressed as the means ± standard error of the means (SEM). Fecal consistency scores and viral RNA titers in rectal fluids were analyzed and compared by a Student's t-test using GraphPad Prism software (GraphPad Prism Inc.). NK cell activity, NK cell numbers, T cell numbers and cytokine amounts were analyzed by one-way ANOVA using GraphPad Prism software. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Suckling pigs had more severe clinical signs, earlier fecal PEDV RNA shedding and higher serum PEDV RNA titers compared to weaned pigs

The suckling pigs showed severe diarrhea and vomiting at PID 1, whereas the weaned pigs showed milder clinical signs starting only at PID 3 (Table 1). The fecal consistency scores were significantly higher in suckling pigs compared to weaned pigs at all time points examined ($P < 0.05$) (Table 1). The fecal shedding PEDV RNA titer was high in suckling pigs starting from PID 1, whereas the weaned pigs started shedding PEDV RNA at PID 3 and shed at significantly higher titers at PID 5 compared to the suckling pigs ($P < 0.05$) (Table 1). The serum PEDV RNA titers were significantly higher in suckling pigs at all time-points sampled (Table 1). The higher severity of disease in suckling pigs coincided with the higher serum PEDV RNA titers in suckling pigs compared to weaned pigs. The MEM only inoculated control pigs had no detectable PEDV RNA in either serum or feces.

3.2. Suckling pigs had undetectable NK cell activity compared to weaned pigs

The suckling pigs had no detectable NK cell activity in blood and ileal MNCs regardless of PEDV infection, whereas the PEDV infected and uninfected weaned pigs had low, but detectable NK cell activity (percentage of target lysis: 0.3–1.3) at PIDs 1 and 5 (Fig. 1A–D).
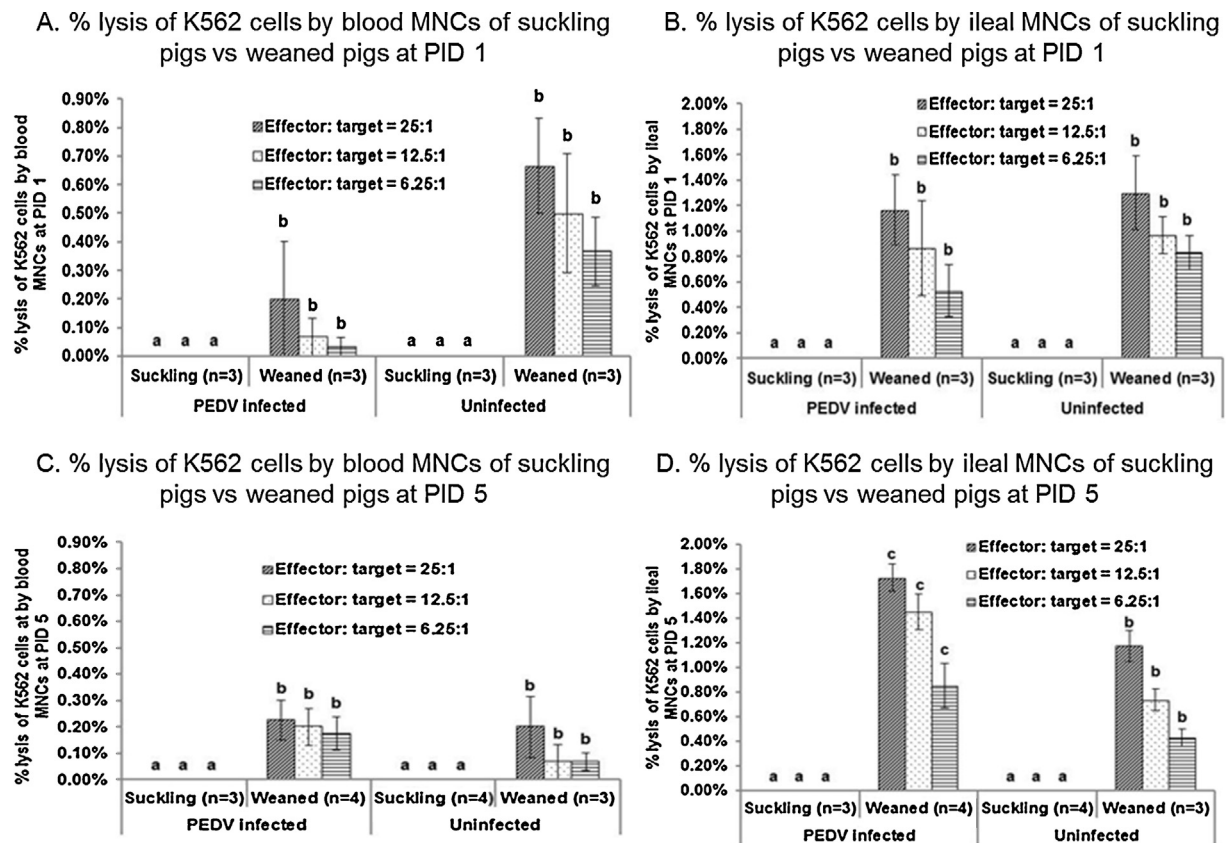
Fig. 1. Suckling piglets had lower NK cell activity (% lysis of K562 cells) compared to weaned piglets in blood and ileal mononuclear cells at PID 1 (A and B) and PID 5 (C and D). Blood and ileal mononuclear cells were co-cultured with CFSE stained K562 cells at indicated ratios overnight. The dead cells were stained by incubating with 7-AAD. The cells were observed by flow cytometry to obtain % of dead K562 cells. The groups that were compared were, suckling uninfected versus weaned uninfected, suckling infected versus weaned infected, suckling infected versus suckling uninfected and weaned infected versus weaned uninfected within a time point. The bar graphs labeled with different alphabetical letters are significantly different ($P < 0.01$).

The infected weaned pigs had significantly increased NK cell activity in ileum at PID 5 compared to uninfected weaned pigs ($P = 0.01$) (Fig. 1D). The NK cell activity was similar for infected versus uninfected weaned pigs at PID 1 (Fig. 1A and B). This coincides with the delayed onset of virus shedding in the infected weaned pigs. The NK cell activity in ileum of uninfected weaned pigs was about 2-fold higher than in the blood of uninfected weaned pigs ($P < 0.01$). The NK cell activity in ileum of infected weaned pigs was about 5-fold higher than in the blood of infected weaned pigs at PID 1 ($P < 0.05$) (Fig. 1A and B) and about 7-fold higher at PID 5 ($P < 0.0001$) (Fig. 1C and D). There was no significant difference in the NK cell activity of blood MNCs of infected and uninfected weaned pigs at PIDs 1 and 5 (Fig. 1A and C). The increase in NK cell activity was not correlated to the increase in NK cell frequencies in the ileal MNCs.

3.3. Weaned infected pigs had significantly higher NK cell frequencies in blood and ileum at PID 5 compared to suckling infected pigs

The uninfected weaned pigs had significantly higher blood NK cell frequencies compared to uninfected suckling pigs ($P < 0.05$) (Fig. 2A). The infected weaned pigs also had significantly higher NK cell frequencies in blood and ileum at PID 5 ($P < 0.05$) compared to infected suckling pigs (Fig. 2A and B). At PID 1, the infected weaned pigs had similar NK cell frequencies in blood to uninfected weaned pigs. At PID 1, the infected suckling pigs showed a significant increase in NK cell frequencies in blood compared to uninfected suckling pigs ($P < 0.05$) (Fig. 2A). The ileal NK cell frequencies were similar across groups at PID 1 (Fig. 2B). However,

at PID 5, the infected weaned pigs had significantly higher NK cell frequencies in ileum compared to infected suckling pigs, although there was an increase in NK cell frequencies in ileum of infected suckling and weaned pigs compared to uninfected suckling and weaned pigs, respectively ($P < 0.05$) (Fig. 2B). The NK cell frequencies of suckling and weaned pigs were higher in blood MNCs than in ileal MNCs at all time-points.

3.4. IFNγ producing CD3-CD4-CD8+ NK cell frequencies were significantly higher in blood of weaned pigs compared to suckling pigs at PID 1 and 5, regardless of PEDV infection status and in ileum at PID 5 for PEDV-infected pigs

Like for the NK cell activity (Fig. 1A and C), the IFNγ producing NK cells were undetectable in blood of suckling piglets (Fig. 3A). The infected weaned pigs had a significantly higher IFNγ producing NK cell frequency in blood than uninfected weaned pigs at PID 1 ($P < 0.03$), although there was no difference between the two groups at PID 5.

The IFNγ producing NK cell frequency in ileum was similar in uninfected/infected suckling (PID 1) and weaned pigs (PID 1, 5) (Fig. 3B). At PID 1, the infected weaned pigs had similar ileal IFNγ producing NK cell frequencies compared to infected suckling pigs. The infected suckling pigs had significantly lower ileal IFNγ producing NK cell frequencies than the infected weaned pigs at PID 5 ($P < 0.01$). At PID 5, the IFNγ producing NK cell frequency was significantly lower in the ileum of infected suckling pigs compared to uninfected suckling pigs ($P < 0.05$). However, in the weaned
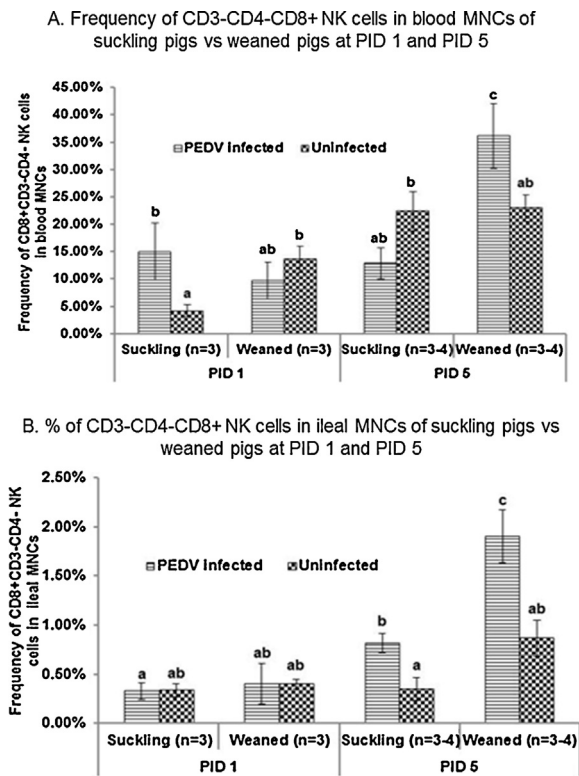
A. Frequency of CD3-CD4-CD8+ NK cells in blood MNCs of suckling pigs vs weaned pigs at PID 1 and PID 5

B. % of CD3-CD4-CD8+ NK cells in ileal MNCs of suckling pigs vs weaned pigs at PID 1 and PID 5

Fig. 2. Weaned infected piglets had higher NK cell frequencies compared to suckling infected piglets in blood (A) and ileum (B) at PID 5. Blood and ileal MNCs were stained with anti-porcine CD3-FITC, CD4-PE and CD8-SPRD. The NK cell (CD3-CD4-CD8+) frequency was expressed as percentage of lymphocytes. The groups that were compared were, suckling uninfected versus weaned uninfected, suckling infected versus weaned infected, suckling infected versus suckling uninfected and weaned infected versus weaned uninfected within a time point. The different time points were also compared within an age group and tissue type. Bar graphs labeled with no common alphabetical letter are significantly different ($P < 0.05$).

A. Frequency of IFNγ positive CD3-CD4-CD8+ NK cells in blood MNCs of suckling pigs vs weaned pigs at PID 1 and PID 5

B. Frequency of IFNγ positive CD3-CD4-CD8+ NK cells in ileal MNCs of suckling pigs vs weaned pigs at PID 1 and PID 5

Fig. 3. IFNγ producing CD3-CD4-CD8+ NK cell frequencies were higher in weaned pigs compared to suckling pigs at PID 1 and 5 in blood (A) and at PID 5 in ileum (B). Blood and ileal MNCs were cultured in E-RPMI with protein transporter inhibitor, Brefeldin A added. The cells were stained with CD3-FITC, CD8-SPRD, and CD4-biotin followed by streptavidin APC as secondary antibody. Samples were stained intracellularly with anti-porcine IFNγ–PE. Flow cytometric analysis was done and the percentage of CD3-CD4-CD8+ NK cells that were also IFNγ positive was calculated. The groups that were compared were, suckling uninfected versus weaned uninfected, suckling infected versus weaned infected, suckling infected versus suckling uninfected and weaned infected versus weaned uninfected within a time point. The different time points were also compared within an age group and tissue type. Bar graphs labeled with no common alphabetical letter are significantly different ($P < 0.05$).

infected pigs there was no significant reduction in the ileal IFNγ producing NK cell frequency compared to uninfected weaned pigs.

3.5. Pro-inflammatory cytokine profiles of PEDV infected suckling pigs differed from PEDV infected weaned pigs and coincided with fecal and serum PEDV RNA titers

There was a marked induction of serum IFNα in infected suckling pigs at PID 1 which declined significantly thereafter. For infected weaned pigs, the response was highest at PID 3 (Fig. 4A). Infected suckling pigs had significantly higher IFNα levels compared to infected weaned pigs at PID 1 ($P < 0.001$). At PIDs 3 and 5, there was no difference in the IFNα induction levels in infected weaned and suckling pigs. Overall, the peak induction of serum IFNα in suckling pigs was at PID 1 and was much higher than the peak induction of IFN-α in weaned pigs which was at PID 3 ($P < 0.01$); both peaks coincided with the peaks of PEDV RNA shedding in feces and serum (suckling pigs) or serum (weaned pigs) and onset of diarrhea. The uninfected suckling and weaned pigs had similar low serum IFNα levels.

Serum IL-12 levels followed a similar trend. IL-12 levels of infected suckling pigs were highest at PID 1, whereas for infected weaned pigs, the induction was highest at PID 3 (Fig. 4B). There was no statistical difference in the peak IL-12 levels between the infected suckling pigs at PID 1 and infected weaned pigs at PID 3. However, serum IL-12 levels were significantly higher in infected suckling pigs compared to infected weaned pigs ($P < 0.001$) at PID 1. The infected weaned pigs had significantly higher serum IL-12
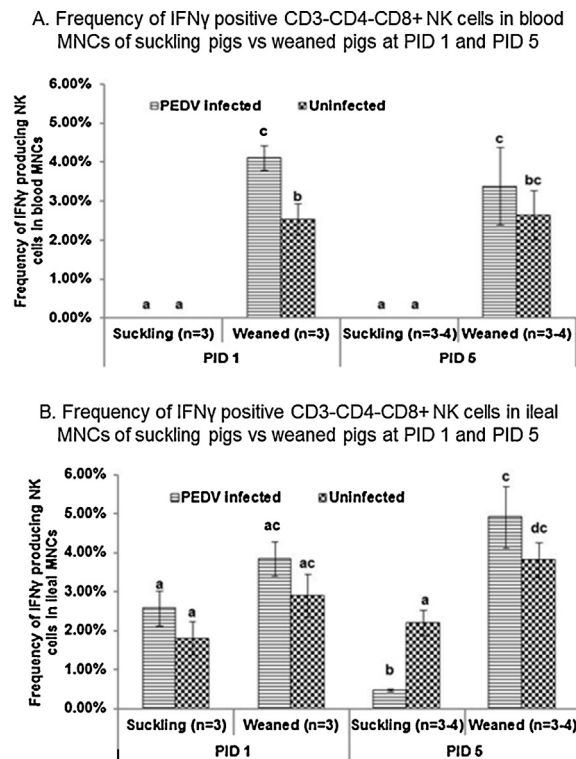
levels at PID 3 compared to infected suckling pigs ($P < 0.01$). The uninfected suckling and weaned pigs had similar serum IL-12 levels at all days.

Overall, serum IL-17 levels were much lower than for all other cytokines measured. Serum IL-17 levels were significantly higher only in infected weaned pigs compared to infected suckling pigs at PID 5 ($P < 0.001$) (data not shown). Otherwise, there were no significant differences in the serum IL-17 between weaned and suckling infected pigs at PIDs 1 and 3. The uninfected suckling and weaned pigs had similar serum IL-17 levels.

Serum IL-8 levels were significantly higher only in infected weaned pigs compared to infected suckling pigs at PID 5 ($P < 0.01$) (Fig. 4C). There was no difference in the serum IL-8 between weaned and suckling infected pigs at PIDs 1 and 3. The uninfected suckling and weaned pigs had similar serum IL-8 levels.

Serum TNFα levels also followed similar trends to IFNα including the PIDs of peak levels in infected weaned and suckling pigs. Infected suckling pigs had highest TNFα levels at PID 1, whereas for infected weaned pigs, the response was highest at PIDs 3 and 5 (Fig. 4D). Serum TNFα was significantly higher in weaned infected pigs than in infected suckling pigs at PIDs 3 and 5 ($P < 0.001$), whereas there was no significant difference in the serum TNFα between infected suckling and infected weaned pigs at PID 1. The peak TNFα level in weaned pigs at PID 5 was significantly higher than the peak TNFα level in suckling pigs at PID 1 ($P < 0.01$).
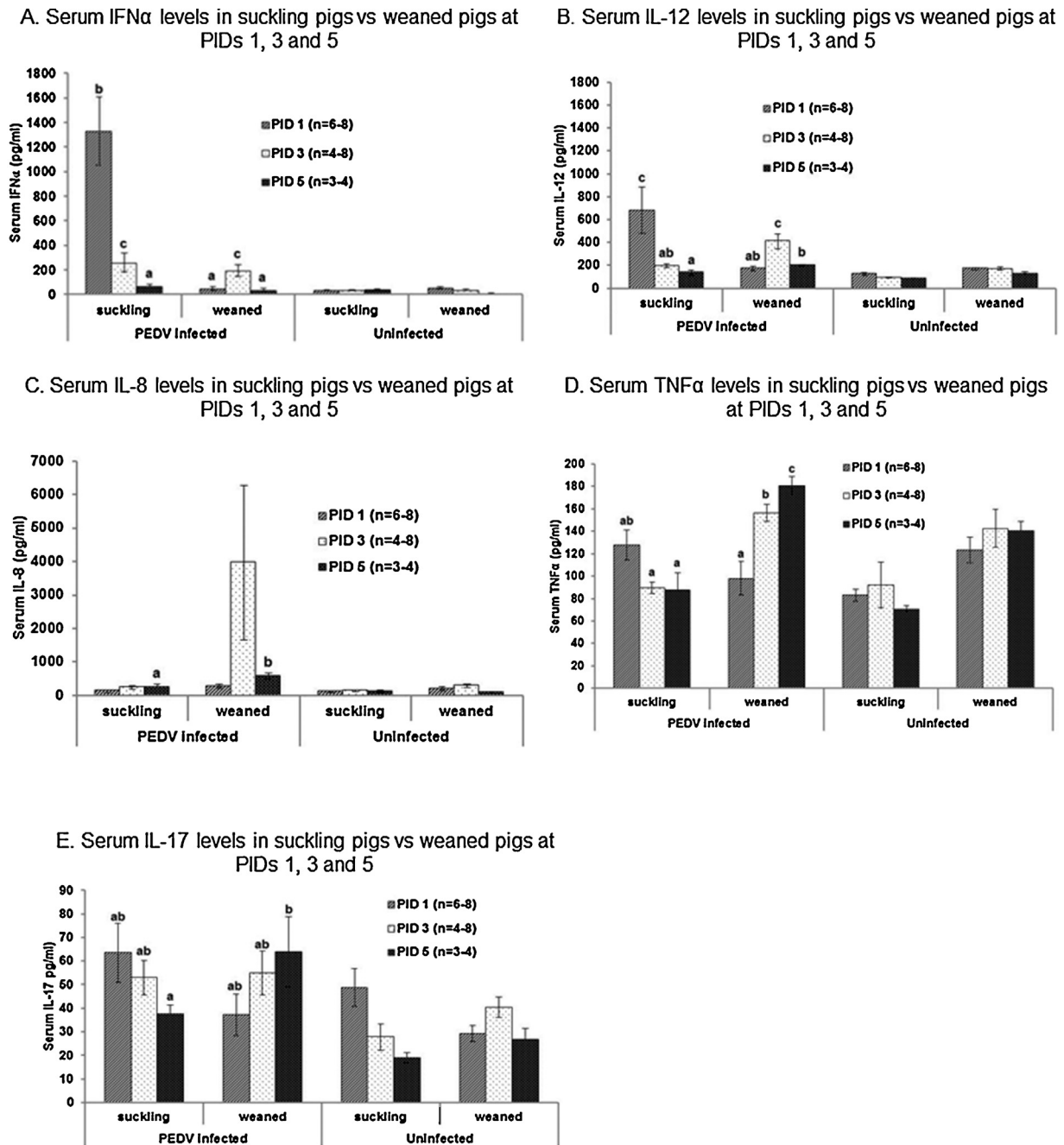
Fig. 4. Inflammatory cytokine profile of PEDV infected suckling piglets differed from PEDV infected weaned pigs and coincided with fecal and serum PEDV RNA. The graphs summarize the serum IFNα (A), IL-12 (B), IL-8 (C), TNFα (D) and IL-17 (E) in suckling and weaned pigs at PIDs 1, 3 and 5. Blood samples were collected from the pigs at the specified time points and sera separated by centrifugation and frozen at −20 °C until use. The cytokine assays were done using standard assays. The groups that were compared were, infected suckling versus infected weaned at different time points, uninfected suckling or weaned versus infected suckling or weaned at different time points. The different time points within infected groups of suckling or weaned pigs were also compared. Bar graphs labeled with no common alphabetical letter are significantly different ($P < 0.01$).

The serum IFNγ, IL-4 and TGFβ levels were measured and there were no differences in their levels between the treatment groups (data not shown).

3.6. CD3+CD4+ T cell frequencies were higher in blood and ileum of suckling pigs than in weaned pigs, whereas there was no difference in CD3+CD8+ T cell frequencies in ileum of suckling and weaned pigs

The CD3+CD4+ T cell frequencies in blood and ileum of uninfected suckling pigs were significantly higher than those in uninfected weaned pigs at PIDs 1 and 5 ($P < 0.01$) (Fig. 5A and B). However, the infected suckling pigs had statistically similar CD3+CD4+ T cell frequencies in blood at PID 1 compared to those of infected weaned pigs, whereas blood (Fig. 5A) and ileum (Fig. 5B) of infected suckling pigs had higher CD3+CD4+ T cell frequencies compared to infected weaned pigs at PID 5 ($P < 0.01$). The CD3+CD4+ T cell frequency in blood did not differ between PIDs 1 and 5 within the infected or uninfected groups of suckling or weaned pigs. The CD3+CD4+ T cell frequencies in blood and ileal MNCs were lower in infected suckling pigs compared to uninfected suckling pigs at PID 1 ($P < 0.01$) (Fig. 5A and B). The frequency
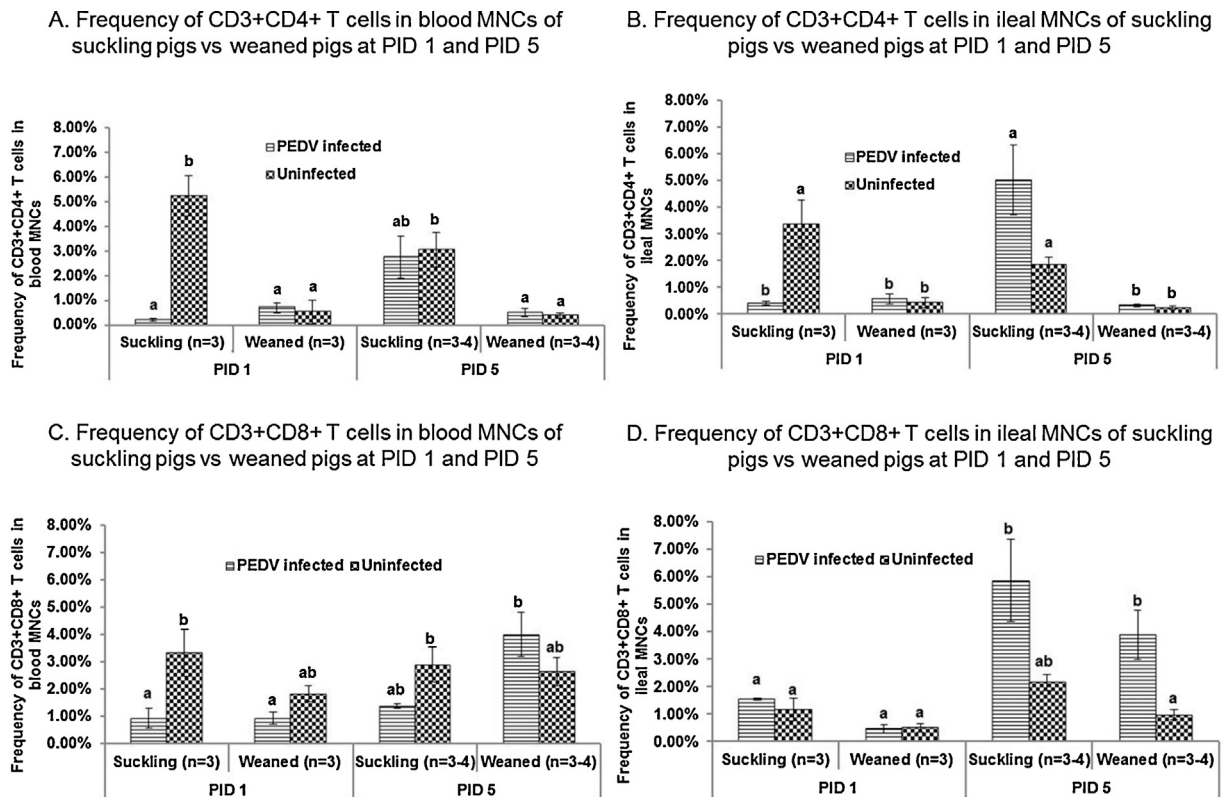
Fig. 5. CD3+CD4+ cells were higher in ileum of suckling pigs than weaned pigs whereas there was no difference in CD3+CD8+ cells in ileum of suckling and weaned pigs. The graphs show the percentage of CD3+CD4+ cells in blood (A) and ileum (B) of suckling and infected pigs at PIDs 1 and 5, and the CD3+CD8+ cells in blood (C) and ileum (D) of suckling and infected pigs at PIDs 1 and 5. Blood and ileal mononuclear cells were stained with CD3-FITC, CD4-PE and CD8-SPRD for 15 min at 4°C. The frequency of T cells or NK cells was expressed as percentage of lymphocytes. The groups that were compared were, suckling uninfected versus weaned uninfected, suckling infected versus weaned infected, suckling infected versus suckling uninfected and weaned infected versus weaned uninfected within a time point. The different time points were also compared within an age group and tissue type. Bar graphs labeled with no common alphabetical letter are significantly different (*P* < 0.05).

of ileal CD3+CD4+ T cells was higher at PID 5 compared to PID 1 for suckling infected pigs (*P* < 0.05). The infected suckling pigs had transient relative leukopenia of CD3+CD4+ and CD3+CD8+ T cells at PID 1 compared to uninfected suckling pigs. There was no difference in the CD3+CD4+ T cell frequency in blood and ileal MNCs in weaned infected and uninfected pigs at either time point.

The CD3+CD8+ T cell frequency in blood MNCs was significantly lower in infected suckling pigs compared to uninfected suckling pigs at PID 1 (*P* = 0.045). The infected weaned pigs had significantly higher frequency of CD3+CD8+ T cells in blood at PID 5 compared to PID 1. There was no difference in the CD3+CD8+ T cell frequency in blood MNCs in infected and uninfected weaned pigs at either time point. There was no difference between the suckling and weaned uninfected pigs in CD3+CD8+ T cell frequencies in blood (Fig. 5C) and ileal (Fig. 5D) MNCs. Suckling infected pigs had significantly lower CD3+CD8+ T cell frequency in blood MNCs compared to weaned infected pigs at PID 5. The CD3+CD8+ T cell frequency in ileal MNCs was similar in uninfected suckling and weaned pigs. There was also no difference in the ileal CD3+CD8+ T cell frequency between the infected weaned and suckling pigs. The CD3+CD8+ T cell frequency in ileal MNCs of infected weaned pigs was significantly higher compared to uninfected weaned pigs (*P* < 0.01) and the same was true for infected and uninfected suckling pigs (*P* < 0.01). The infected suckling pigs had significantly higher frequency of CD3+CD8+ T cells in ileum at PID 5 compared to PID 1. Similarly, infected weaned pigs had significantly higher frequency of CD3+CD8+ T cells in ileal MNCs at PID 5 compared to PID 1 (*P* < 0.05).

4. Discussion

In the present study, we investigated the differences between suckling and weaned pigs in innate immune responses to PEDV infection and attempted to assess if these differences coincide with the greater severity of PED in suckling pigs. The major findings of this study were: (1) Suckling pigs had earlier onset and more severe diarrhea and earlier fecal and higher serum PEDV RNA titers compared to weaned pigs; (2) PEDV infected and uninfected suckling pigs had lower NK cell frequencies and no activity and lower IFNγ producing NK cell frequencies compared to weaned pigs; (3) IFNα and pro-inflammatory cytokine profiles of PEDV infected suckling pigs differed from PEDV infected weaned pigs. In infected suckling and weaned groups, peak IFNα, IL-12 and TNFα levels coincided with onset of diarrhea and fecal PEDV RNA shedding and peak serum PEDV RNA titers (PIDs 1 and 3, respectively); and (4) Frequencies of CD3+CD4+ T cells were significantly higher in ileum of suckling pigs than in weaned pigs, whereas there was no difference in frequency of CD3+CD8+ T cells. There was a transient relative leukopenia in CD3+CD4+ and CD3+CD8+ T cells in blood of suckling, but not weaned pigs at PID1.

The nursing pigs showed severe clinical signs and fecal and serum PEDV RNA as early as PID 1 as reported previously (Jung et al., 2014; Stevenson et al., 2013), but the weaned pigs showed mild and delayed clinical signs and delayed shedding of PEDV RNA in feces, in agreement with previous findings (Madson et al., 2014). In general, neonates of various species are more susceptible to severe disease than adults (Camacho-Gonzalez et al., 2013; Rose, 1983; Rosenberg et al., 1981; Tregoning and Schwarze, 2010; Wohlfender et al.,

2009). One of the reasons for the high susceptibility of neonates to severe disease from infections is deficient innate and adaptive immune responses/memory (Levy, 2007; Levy et al., 1999). In the absence of previous exposure to pathogens, and therefore no adaptive immune responses, the newborn depends on innate immune responses to clear or reduce viral infection. In instances when the virus infection overwhelms the already weak innate immunity of the neonate, severe disease results (Firth et al., 2005). The virus levels in blood were directly related to severity of certain enteroviral diseases in infants (Dagan et al., 1985; Yen et al., 2007) as well as in other viral infections (Maggi et al., 2003; Vaughn et al., 2000). Interestingly in the present study, the moderate titer of PEDV RNA in serum of suckling pigs and 100-fold lower titers in weaned pigs coincided with the greater severity of disease in suckling pigs compared to weaned pigs. The severity of disease in suckling pigs also coincided with the early high titer PEDV RNA fecal shedding.

Porcine NK cells are identified as CD3-CD8+ cells (Gerner et al., 2009). Suckling pigs had no detectable NK cell activity in blood or ileal MNCs and the activity did not change with infection, although the suckling pigs had comparable NK cell frequencies in most instances (except PIDs of infected pigs) to weaned pigs. This is in confirmation of previous studies of NK cell frequency and cytotoxic activity of lymphocytes of healthy suckling pigs against K562 cells (Onizuka et al., 1987; Yang et al., 1987). Newborn pigs also did not have NK cell mediated cytotoxic activity against PK-15 cells persistently infected with TGEV (Cepica and Derbyshire, 1984). Studies of TGE showed that vaccination with a modified live TGEV vaccine did not increase NK cell activity in newborn pigs (Raymond and Wilkie, 1998), but in vitro stimulation of adult monocytes increased NK cell activity (Charley et al., 1983). In the present study, PEDV infected weaned pigs showed increased frequencies as well as activity of NK cells in the ileum. Although uninfected suckling pigs had comparable NK cell frequencies to weaned pigs, their frequencies were reduced in blood and slightly increased in ileum following infection, but NK cell activity remained undetectable. Studies of human infants showed that neonates had significantly lower NK cell lytic activity and the activity was further reduced by infection or sepsis (Georgeson et al., 2001; Uksila et al., 1982). NK cells of infants also had reduced antiviral activity such as defective killing of virus infected cells and reduced cytokine secretion (Jacobson et al., 2013). Collectively, reduced NK cell activity and NK cell frequencies may have contributed to the early onset of virus shedding and the severe clinical signs in the suckling versus weaned pigs.

Coinciding with NK cell cytotoxic activity, the weaned pigs also had higher frequency of IFNγ positive NK cells compared to suckling pigs. Further, PEDV infection reduced the frequencies of IFNγ producing NK cells in ileum of suckling pigs. Neonatal MNCs are deficient in IFNγ production in response to antigenic stimuli (Wilson et al., 1986). NK cells are the major source of IFNγ and activation of NK cells by IFNγ is essential for their lytic activity (Wang et al., 2012). Studies have shown that the increase in frequency, activity of NK cells and IFNγ production by NK cells is essential for reduction of viral load in mice infected with lymphocytic choriomeningitis virus or influenza virus (Biron et al., 1983; Mack et al., 2011; Stein-Streilein et al., 1988). Further, production of IFNβ and IFNγ in intestine of mice was essential and sufficient to induce innate immune cells to eliminate certain bacterial pathogens in mice (Sotolongo et al., 2011). Since the suckling pigs showed reduced IFNγ positive NK cell frequencies, this could result in reduced viral clearance contributing to the enhanced disease. Similar to our observations, foot and mouth disease virus decreased the lytic activity of NK cells in swine (Toka et al., 2009). Studies showed that IL-18 production stimulates IFNγ production and IL-18 production is less in intestinal epithelium of neonatal pigs compared to older pigs (Muneta et al., 2002). Thus, it appears that the failure to induce sufficient IFNγ production by NK cells might

be one reason for increased severity of disease and higher systemic load of PEDV in suckling pigs.

The weaned pigs had a delayed pro-inflammatory cytokine induction compared with suckling pigs which coincided with delayed infection, disease and shedding of PEDV RNA in feces of weaned pigs. IFNα is an antiviral cytokine produced in response to viral infection by monocytes and dendritic cells (Hansmann et al., 2008; Siegal et al., 1999). Consistent with fecal virus shedding and serum viral RNA titers, the suckling pigs had the highest serum IFNα induction at PID 1. The higher systemic IFNα response in suckling pigs at the early stage of infection coincides with the serum PEDV RNA titer and very severe infection. Collectively, lack of NK cell activity, reduced NK cell frequency and IFNγ production might have contributed to the early onset of viral shedding, viremia (viral RNA in serum) and severity of clinical signs. This is similar to the IFNα induction by TGEV infection in suckling pigs (La Bonnardiere and Laude, 1981). Notably, treatment of newborn pigs with the interferon inducer, polyinosinic: polycytidylic acid complexed with poly-L-lysine, resulted in a delayed onset of clinical signs when pigs were infected with TGEV (Lesnick and Derbyshire, 1988) and the IFNα treatment enhanced NK cell activity (Charley et al., 1985). Also, treatment of TGEV infected suckling pigs with oral human IFNα increased their survival rates (Cummins et al., 1995). In vitro studies of human dendritic cells stimulated with viruses show that neonatal cells are capable of producing IFNα responses similar to those of adults (Renneson et al., 2009). The increased IFNα may be the result of infection in suckling pigs in the early stages and may have led to the reduced PEDV RNA shedding in feces at a later stage compared to the weaned pigs.

The present studies showed that weaned infected pigs had higher IL-8 at PID 5 and higher TNFα induction at PIDs 3 and 5 compared to suckling pigs. TNFα is a proinflammatory cytokine secreted by activated macrophages, NK cells, T cells and other cells (Bradley, 2008). Serum levels of TNFα are often associated with severity of disease (Jiang et al., 2003; Waage et al., 1987). Studies of rotavirus infected children show that higher TNFα and lower IFNγ are associated with more severe symptoms of infection (Jiang et al., 2003). Similarly, studies of human rotavirus infection in a gnotobiotic pig model show that levels of cytokines like IFNγ and TNFα are higher when pigs are infected with virulent virus compared to attenuated virus (Azevedo et al., 2006). Human neonatal monocytes/macrophages stimulated in vitro with bacterial lipopolysaccharides or virus showed reduced TNFα responses when compared to monocytes/macrophages from adults (Levy et al., 2004; Valero et al., 2014). In the present study, although the suckling pigs had more severe disease, their TNFα levels were similar to weaned pigs at PID1 and less than weaned pigs at PIDs 3 and 5.

IL-8 is a chemoattractant for neutrophils and is secreted by various cell types (Bickel, 1993). Pigs infected with porcine respiratory and reproductive syndrome virus that subsequently cleared the infection had higher IL-8 than pigs that did not clear the infection (Kim and Chae, 2003). Therefore, the higher IL-8 in weaned pigs may have contributed to lower severity of disease. Similar to the pattern of IFNα, the serum levels of cytokines IL-17 and IL-12 were high in suckling pigs at PID 1 and in weaned pigs at PIDs 3 or 5 and coincided with the delay in onset of clinical signs and fecal PEDV RNA shedding in weaned pigs. Studies of human neonates show that they are capable of producing certain pro-inflammatory cytokines in comparable levels to adults (Schnurr et al., 2005). Therefore, suckling piglets could have a similar ability to produce these cytokines as the weaned pigs. Thus, pro-inflammatory cytokine profiles of suckling and weaned pigs mirrored the severity of infection and viremia. The suckling pigs lacked innate immune activity to delay onset of viral shedding and viremia that resulted in more cells infected and higher IFNα (Jung et al., 2015a). In

contrast, the robust innate immune activity in weaned pigs may have delayed the onset of viral shedding and viremia, resulting in a lower IFNα response. However, the innate immune response was not enough to prevent the higher fecal viral shedding at PID 5 in weaned pigs although there was reduced diarrhea compared to suckling pigs. The results also indicated the ability of suckling pigs to produce proinflammatory cytokines comparable to weaned pigs.

CD4+ T cells represent T helper cells that aid in the humoral immune response and are responsible for Th2 cytokine production. CD8+ T cells represent cytotoxic T cells that are important in killing virus infected cells (Germain, 2002; Gerner et al., 2009). The CD3+CD4+ T cell frequency was higher in the uninfected suckling pigs compared to uninfected weaned pigs, whereas infected suckling pigs had transient relative leukopenia at PID 1 and then had increased CD3+CD4+ T cell frequencies at PID 5 compared to uninfected suckling pigs. Neonates have a polarity toward Th2 responses dominated by CD3+CD4+ T cells and this changes when they are given viral vaccines and it also changes with age (Dowling and Levy, 2014; Kelly et al., 2007; Kovarik and Siegrist, 1998; Siegrist et al., 1998). The present study confirmed that the neonatal swine intestine has increased CD3+CD4+ T cells that can be altered by viral infection. The CD3+CD8+ T cell frequency was comparable in suckling and weaned uninfected pigs and infection increased the intestinal CD3+CD8+ T cell frequency in both groups. Thus, the observed differences in PEDV infectivity between suckling and weaned pigs might not be influenced by early CD3+CD8+ T cell frequency. Since CD3+CD8+ T cells are involved in early antiviral adaptive immune responses, these cells are expected to play important roles in PEDV infection of the intestine. Longer observations are required to determine if there are differences in adaptive immune responses between the suckling and weaned pigs.

In summary, deficiency in innate immune function of NK cells may have contributed to the higher severity of PEDV infection in suckling versus weaned piglets. Interventions to enhance the NK cell activity and innate immunity may reduce the morbidity and mortality associated with PEDV in suckling piglets. Further studies are required to examine this possibility.

Conflict of interest

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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