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Pigs' intestinal barrier function is more refined with aging

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ABSTRACT

The high mortality upon enteric virus infection in piglets causes huge economic losses. To control these infections, potential causes for this high susceptibility for enteric virus infections in younger piglets were analyzed by comparing the intestinal barrier between 1-week, 2-week and 4-week-old piglets. In this study, histological staining was used to analyze morphological differences in intestinal villi, real-time qPCR was performed to assess mRNA expression levels of genes that were related to viral infection and differentiation of immune cells, and flow cytometry was utilized to measure the frequencies of T cells. According to the results obtained, 1-week-old piglets have intestinal villi with shallower crypts, less well developed epithelial cells and a more immature immune system compared to older pigs. Moreover, high amounts of enteric virus invasion-assisting proteins but low amounts of resistant proteins in 1-week piglets could also be a reason for the high susceptibility of 1-week-old piglets.

1. Introduction

Piglet diarrhea is one of the major causes of high morbidity and mortality among piglets, and is responsible for serious economic losses in the pig industry (Katsuda et al., 2006). Nowadays, bacterial and parasitic diseases that cause diarrhea can be effectively controlled, but diarrhea due to viral infections remains a large problem. This is especially the case in piglets where enteric viruses tend to be highly pathogenic and even lethal. At present, enteric viruses known to cause high mortality in piglets include alphacoronaviruses like transmissible gastroenteritis virus (TGEV), porcine epidemic diarrhea virus (PEDV), swine enteric alphacoronavirus (SeACoV) and deltacoronavirus like porcine deltacoronavirus (PDCoV) (Pan et al., 2017; Wang et al., 2019a; Yang et al., 2020a; Koonpaew et al., 2019). Also, porcine rotavirus (PoRV), belonging to the genus of rotavirus, is considered a major cause of diarrhea in piglets (Chepngeno et al., 2019). In our previous study, we observed that within the same time period, 1-week-old pigs were most susceptible to PEDV resulting in high mortality, while 2-week-old pigs had no clinical symptoms, and 4-week-old pigs could not be infected (Yang et al., 2020b). However, why these enteric viruses are only highly pathogenic and lethal in piglets and not in adult pigs is currently not known and this was the main focus of this study. We hypothesize that the intestinal barrier of pigs at different ages correlates with susceptibility to enteric viral invasion infection.

The intestinal barrier with its villi, microbiota and immunological tissue is responsible for the absorption of nutrients and is involved in resistance to pathogens (Turner, 2009). From the physiological structure, it is clear that the intestinal villi serve as the first barrier since these come into direct contact with the intestinal dietary compounds, commensal bacteria and pathogens. The length of the villi and depth of the crypts are important hallmarks for intestinal development and health (Jayaraman et al., 2013). Furthermore, the mucus layer on top of the villi which is secreted by mucin2^{+} goblet cells is also involved in protection against toxins and pathogens (Kim and Ho, 2010). A defect in goblet cells leads to a disorder of the mucus layer, which in turn promotes the adhesion of pathogenic organisms and results in enhanced susceptibility to acute PEDV infection (McElroy et al., 2011; Liu et al., 2015a; Jung and Saif, 2015). Intestinal CK18^{+} (Cytokeratin 18) microfold cells recognize some intestinal bacteria, transport antigens to immune cells in the lamina propria and stimulate the induction of an

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immune response (Gebert et al., 1996). Paneth cells, marked as lysozyme^{+} cells in intestines, mainly secrete defensins. These defensins contribute to the antimicrobial action of granulocytes and are thus involved in the host defense in the mucosa of the small intestine (Yokoi et al., 2019; Ganz, 2003). Moreover, some proteins facilitate viral infection. These include the enteric virus invasion-assisting proteins TGEV invasion receptor pAPN (porcine Aminopeptidases), tight junction protein Occludin, cell proliferation marker Ki67. Other proteins inhibit enteric virus infection, include EGF (Epidermal Growth Factor), cellular repair cytokine IL-22 and pBD2 (porcine β-defensin) (Liu et al., 2015b; Luo et al., 2017; Tang et al., 2016; Sales Gil and Vagnarelli, 2018; Zenewicz and Flavell, 2011). The ablation of the expression of these proteins in epithelial cells also regulates the susceptibility to enteric virus infection. Moreover, intestinal microbes also regulate invasion of intestinal pathogens (Ubeda et al., 2017). For example, probiotics could provide a health benefit to the host (Kober and Bowe, 2015) while pathogenic bacteria like *shigella* would destroy intestinal health (Nhieu and Sansonetti, 1999).

In addition to the intestinal epithelium and intestinal content, also the mucosal immune system in the lamina propria can provide protection against over 90% of intestinal pathogens. During the adaptive immune response, proliferation and differentiation of B and T cells is required. As reported, the cytokines IL-2, IL-6, IL-16 promote help for T cell differentiation and survival (Malek, 2003; Richmond et al., 2014; Li et al., 2018), IL-21 promotes B cell proliferation, differentiation and survival (Ettinger et al., 2008). IL-7 is involved in T and B cell receptor V(D)J recombination and the maintenance of naive T and B cells (Mazzucchelli and Durum, 2007).

In newborn piglets, the adaptive immune system is not well developed yet (Akdis et al., 2016; Westrom et al., 2020). Therefore, when piglets are infected with enteric viruses, the innate immune response and especially production of anti-viral cytokines are crucial in the antiviral response. Type I interferons (IFN) were first recognized for blocking viral replication and subsequent infection (Decker et al., 2002). During enteric virus infection in pigs, IFN-λ was reported to induce more robust antiviral activity compared to IFN-α (Li et al., 2017), and the type II interferon IFN-γ was reported to be involved in the induction of an inflammatory response rather than showing direct antiviral activity (Lee et al., 2017). Both types of responses are initiated by pattern-recognition receptors (PPRs), of which toll-like receptors (TLRs) are one of them. Viruses can induce downstream immune responses by stimulating TLRs, and induce up-regulation of interferon and/or inflammation (Vijay, 2018). Therefore, expression level of IFNs, the upstream toll-like receptors (TLRs) of IFNs (Perkins and Vogel, 2015) and production of inflammatory cytokines (Chen et al., 2018) were also suspected to influence the outcome of enteric viruses infection in piglets.

To further investigate potential mechanisms that may explain why young piglets are more susceptible to enteric virus infections than adult pigs, a comparison of the physiological structure of the intestine, the abundance of intestinal microbiota, frequencies of T and B cells, levels of IFNs and inflammatory cytokines, and the expression of epithelial molecules related to enteric virus invasion between newborn piglets and older pigs was performed. These outcome of this study may contribute to future possible control strategies against enteric virus infection in piglets.

2. Materials and methods

2.1. Animal experiments

Healthy pigs were screened by RT-qPCR using primers that are specific for common enteric viruses in pigs (Ding et al., 2020). In total, 9 piglets (Duroc-Landrace-Yorkshire) including 3 piglets from each age group (1-week-old, 2-week-old, and 4-week-old) were purchased from a pig farm located in Dingxi, Gansu province and housed in the same animal rooms. The biological sex of pigs was chosen randomly. After euthanizing pigs, tissues were collected and stored in Trizol reagent at −80 °C for RNA isolation. All experimental procedures and animal care protocols were approved by the guidelines for Care and Use of Laboratory Animals of Lanzhou Veterinary Research Institute (LVRI), Chinese Academy of Agricultural Sciences, China.

2.2. Histological analysis

After euthanizing pigs, different segments of intestines (duodenum, jejunum and ileum) were collected, fixed for 24 h in 10% formalin, dehydrated according to a standard protocol and then embedded in paraffin. Next, 5–10 μm sections were cut from each tissue block, deparaffinized in xylene, stained with Hematoxylin and Eosin (H&E) and analyzed using an optical microscope (Olympus. Japan) to evaluate villi area, crypt depth, and the average number of intestinal lymphoid nodes.

2.3. Real-time PCR

Intestinal tissues were thawed and homogenized in pre-chilled Trizol reagent (Invitrogen) containing 0.5 mm silicon beads with a bead-beater (Eppendorf) and then centrifuged at 12,000 rpm for 10 min at 4 °C. Total RNA from each sample was first treated with gDNA wiper to remove genomic DNA and then reverse transcribed into cDNAs using random primers. cDNAs were applied to real-time qPCR analysis for gene expression detection using the ABI 7500 system with SYBR® Green-based qPCR Supermix (Novogene Technology Co., Ltd). Detailed information onthe primers is shown in Table 1. An amplification was carried out with a denaturing step at 95 °C for the 30s, followed by 40 cycles at 95°s for 10s and 60 °C for 30s.

2.4. Western blotting

Total protein lysate was prepared using RIPA lysis buffer (Beyotime, China) containing protease inhibitors. The protein was loaded on a x% polyacryl amide gel for SDS-PAGE, transferred to PVDF membrane (Merck, U.S.), and immunoblotted overnight at 4 °C in the presence of the specific primary antibodies. Next, the membrane was washed and incubated with specific secondary antibodies. Finally, the immunoblots were developed with chemiluminescence detection reagent. (Advansta, U.S.).

2.5. Isolation of peripheral blood mononuclear cells and lymphocytes of tissues

Fresh blood was collected through the anterior vena cava and immediately mixed with anticoagulant and diluted with PBS for isolation of PBMCs (peripheral blood mononuclear cells). PBMCs were isolated by density gradient centrifugation using Percoll Plus (GE Healthcare) at 400×*g* for 30 min at room temperature. The middle layer after centrifugation contained the PBMCs and was collected. To remove any erythrocytes that were still present after the Percoll isolation, erythrolysis was applied. Next, cells were washed with PBS for three times and prepared to be stained with antibodies for flow cytometry analysis. For the isolation of lymphocytes from peyer's patches (PPs) and mesenteric lymph node (MLN) (without capsule) in pigs, tissues was collected and subsequently washed repeatedly with PBS to remove of intestinal content. Next tissues were minced with scissors, and meshed through a 40 μm cell strained to obtain single intestinal cells. Next, density gradient centrifugation with Percoll was performed to acquire lymphocytes (middle layer). Afterwards, these lymphocytes were washed three times, calculated, and 2×10^6 cells were stained for flow cytometry analysis.

Table 1
Primers for real-time PCR.

Names	Forward primers	Reversed primers
Occludin	CAGGTGCACCCTCCAGATTG	ATGTCGTTGCTGGGTGCATA
IFN-γ	TGGTAGCTCTGGGAAACTGAATG	GGCTTTGCGCTGGATCTG
NOD1	ACTGACAGTGGGGTGAAGGT	TTTCCCAGTTTCAGGCACTTG
Ki67	CCTGAATCCGCAAGAAGATGCTAAG	GACAGTCTCAATCTTGTAACAGTGC
Lgr5	GAGCCTGGGAAAGCAAACC	GGACAAATGCCACGGAAGA
CK18	AGTTCTGTGGACAATGCCCG	CATCAATGACCTTGCGGAGC
Lysozyme	GGTCTATGATCGGTGCGAGT	AACTGCTTTGGGTGTCTTGC
pDB2	GCTGCTGACTGTCTGCCTCCTCTCT	CTGTTGAAGAGCGGGCAGGGGGAGA
Mucin2	GAGGAGAAGTGTGACGACCCCGA	CGGCGTGGGAGCACTGGCGGGAG
APN	AAGGGATTCTACATTTCCAAGGC	GAAGTAGGAATCAGGCAACAGCG
EGF	TCTGAACCCGGACGGATTTG	GACATCGCTCGCCAACGTAG
IL-1β	CAGCCAGTCTTCATTGTTCAGGTT	AGATTTGCAGCTGGATGCTC
IL-2	GTGAATATGATGATGAGACAGTAA	CAAGTCAGTGTTGAGTAGATG
IL-6	AATGCTCTTCACCTCTCC	TCACACTTCTCATACTTCTCA
IL-16	AATGCTCTTCACCTCTCC	TCACACTTCTCATACTTCTCA
IL-7	ATCCTTGTTCTGTTGCCAGTAGC	AAAAAGTTAGGTTCGTTATTCAG
IL-21	ATGGAGAAAATAGTCATCTGCCT	TCTCCCGTATTTGCTGACTTTAG
IL-22	GATGAGAGAGCGCTGCTACCTGG	GAAGGACGCCACCTCCTGCATGT
IFN-λ	GTCCCTCTTGGAGGACTGGA	TGCTGTGCAGGGATGAGTTC
IFN-β	CGATACCAACAAAGGAGCAGCAA	CATCTCGTGGATAATCAATACTG
TLR2	TCACTTGTCTAACTTATCATCCTCTTG	TCAGCGAAGGTGTCATTATTGC
TLR3	AGTAAATGAATCACCCTGCCTAGCA	GCCGTTGACAAAACACATAAGGACT
TLR4	GCCATCGCTGCTAACATCATC	CTCATACTCAAAGATACACCATCGG
TLR9	AGGGAGACCTCTATCTCCGC	AAGTCCAGGGTTTCCAGCTT
IL-17	AAGTCCAGGATGCCCAAA	CGGTTCAAGATGTTCAAGTTG
RIPK2	GTGGATGGGCACAAAATCCAG	TGGAAGCACTTTGCAACTTTGT
IL-23	CCTTCTCCGCCTCAAGATCC	TACTGGCTCAGAGTTGCTGC
TNF-α	GTCTCAAACCTCAGATAAG	GTTGTCTTTCAGCTTCAC
GAPDH	CATCCATGACAACTTCGGCA	GCATGGACTGTGGTCATGAGTC

2.6. Flow cytometry

Next, 10^7 lymphocytes isolated from blood, peyer's patches, and MLNs were stained with the mouse anti-porcine monoclonal antibody CD21-PE (Southern Biotech, Cat.No.4530-09) to detect $CD21^+$ B cells. Alternatively, lymphocytes were stained with mouse anti-porcine CD3e-APC (Southern Biotech) together with mouse anti-porcine monoclonal antibody CD4-FITC (Southern Biotech) or mouse anti-porcine monoclonal antibody CD8a-FITC (Southern Biotech) to distinguish $CD3^+CD4^+$ or $CD3^+CD8^+$ cells. Lymphocytes were incubated with antibodies for 30 min at 4 °C, then cells were washed with PBS for three times, resuspended in 100ul PBS, and 10^4 cells were collected for analysis using the C6 flow cytometry machine (BD Accuri™).

2.7. Intestinal microbiota analysis

According to the protocol in the previous experiment (Yang et al., 2020b), high-throughput sequencing was used to detect the distribution and abundance changes of intestinal microbiota in pigs infected with the enteric virus at different weeks of age compared to the healthy pigs. In this study, the data from the previous study was reanalyzed and then absolute abundance changes of intestinal microbes of healthy pigs in different ages were showed.

2.8. Data analysis

All data were analyzed by SPSS. Significant differences were analyzed by one-way ANOVA ("***" means $p < 0.001$, "**" means $p < 0.01$, "*" means $p < 0.05$). Data presented are Mean ± SEM. All figures were generated using the GraphPad prism software 5.01.

3. Results

3.1. Longer villi with shallower crypt depth in 1-week piglets compared to older pigs

In order to observe possible differences in the intestinal villi between pigs of different ages, HE staining was used to evaluated the villi length, crypt depth and presence of lymphoid nodes in all parts of the small intestine (duodenum, jejunum, ileum). As shown in Fig. 1, in 1-week piglets, villi were longer but narrower with shallower crypts compared to 2-week and 4-week-old pigs (Fig. 1A–F). Next, the number and area of lymphoid nodes in the ileum were measured to investigate whether the structure of the villa is able to affect the development of lymphoid nodes. As expected, a significant increase in both numbers and areas of lymphoid nodes were observed in older pigs (Fig. 1G and 1H). Taken together, these results showed that 1-week-old pigs have longer intestinal villi, which are more narrow with crypts that are shallower compared to older pigs. Also lower numbers of lymph nodes and smaller lymph nodes were observed in the ileum of young compared to the older pigs.

3.2. Reduced function of epithelial cells in 1-week piglets compared to older pigs

To further investigate the disparity in the number of functional epithelial cells in intestinal villi from pigs of different ages, expression levels of *mucin2*, *ck18*, and *lysozyme*, markers that are associated with functional intestinal epithelial cells, were analyzed in different intestinal segments by both RT-qPCR and Western-blotting. RT-qPCR results showed high expression levels of *mucin2* in both jejunum and ileum in 2-week and 4-week pigs, compared to 1-week-old pigs (Fig. 2A, 2B, 2C). Meanwhile, in 4-week-old pigs higher mRNA levels of *lysozyme* were observed in the duodenum compared to the other two age groups (Fig. 2A). The Western-blot results demonstrated significantly higher protein levels of mucin2, ck18, and lysozyme in all intestinal segments in 4-week-old pigs compared to 1- and 2-week-old pigs (Fig. 2B, 2D, 2F). Collectively, these data suggest that the intestinal epithelial cells are less well developed in 1-week-old pigs than in older piglets.

3.3. Higher amount of enteric virus invasion-assisting elements in 1-week piglets

After studying the physical structure and distribution of functional

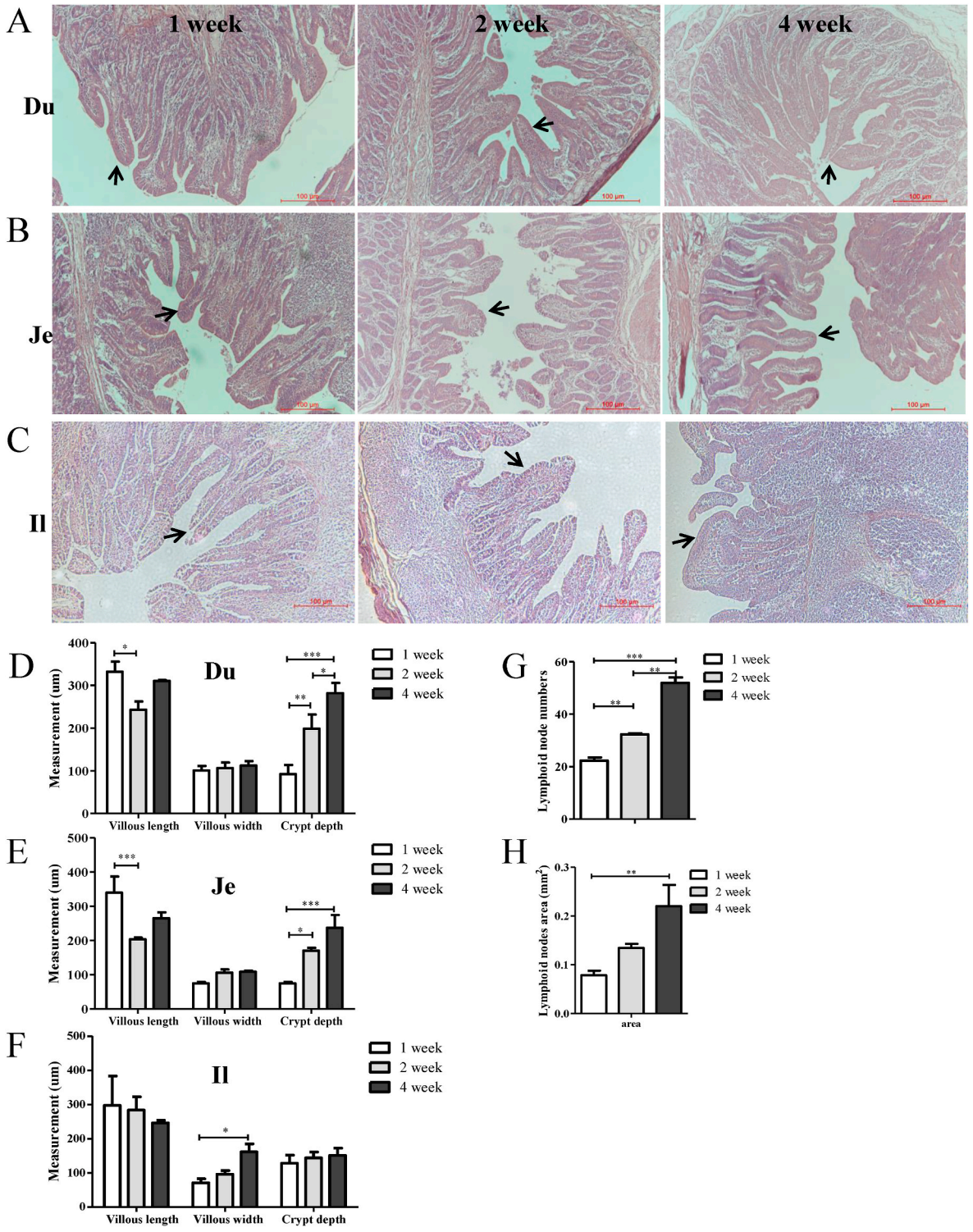

Fig. 1. Longer villi with shallower crypt depth in 1-week piglets compared to older pigs. Hematoxylin-eosin staining was used to study possible differences in the morphology of intestinal villi in duodenum (A), jejunum (B), ileum (C). Villus length, villus width and crypt depth in duodenum (D), jejunum (E), ileum (F) were counted. Numbers (G) and area (H) of lymphoid tissue (nodes) were counted. Arrows point to the representative villi in pigs of different ages. Values reflect the average ± SEM (n = 3). Significant differences are indicated by ***$p < 0.001$, **$p < 0.01$, and * $p < 0.05$.

epithelial cells of intestinal villi, expression levels of specific molecules that are known to influence enteric viral infection were determined. First, expression levels of *occludin*, *pAPN* and *Ki67*, which facilitate enteric viral infection, were measured. As shown in Fig. 3, expression of *occludin* and *pAPN* was much higher in all intestinal segments of 1-week piglets compared to older pigs. In the duodenum no differences in expression level of Ki67 were observed between pigs of different ages, but both in jejunum and ileum Ki67 expression levels gradually decreased with pigs of increasing age (Fig. 3A, 3B, 3C). Besides, expression levels of *EGF*, *IL-22* and *pBD2*, which are critical cytokines to promote epithelial cell growth, maintain cellular repairment and defend against intestinal pathogens, were also evaluated. In general, higher

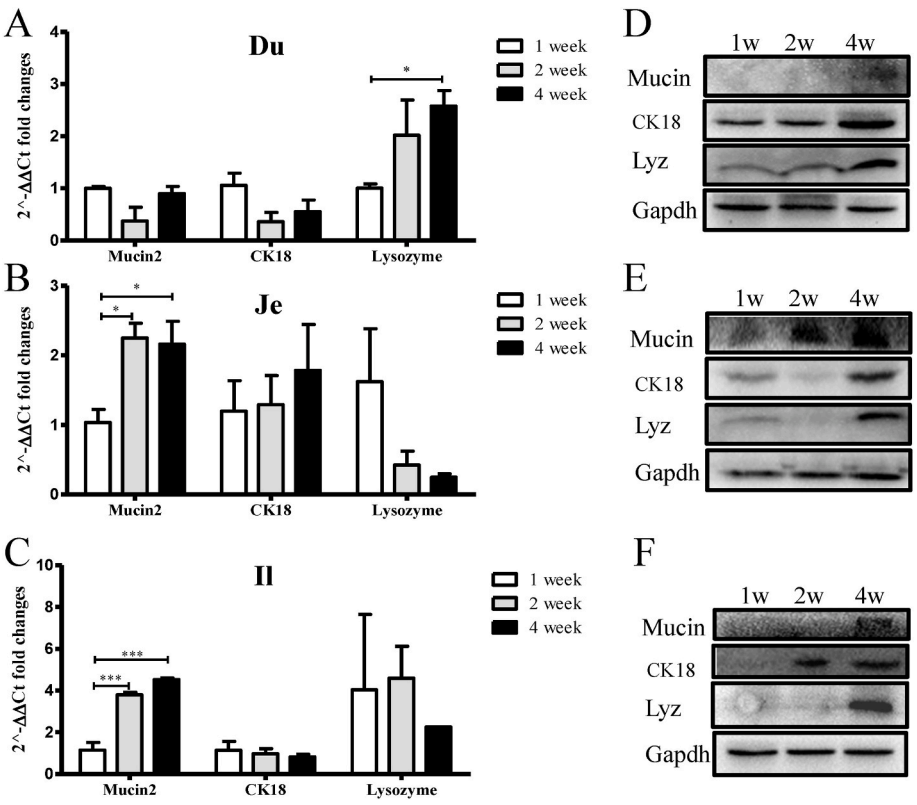

Fig. 2. Reduced function of epithelial cells in 1-week-old piglets compared to older pigs. Real-time PCR was used to detect mRNA expression levels of *mucin2* for goblet cells, *ck18* for microfold cells, *lysozyme* for Paneth cells in the duodenum (A), jejunum (C) and ileum (E). Western blotting was used to identify protein levels of mucin2, ck18 and lysozyme in duodenum (B), jejunum (D) and ileum (F) relative to GAPDH as a reference protein. Significant differences are indicated by $***p < 0.001$, $**p < 0.01$, and $* p < 0.05$.

expression levels of these cytokines were observed in 4-week pigs than younger piglets (Fig. 3D, 3E, 3F). In jejunum, expression levels of IL-22 were increased in 4-week pigs compared to younger pigs, although no significant differences were observed. In the ileum, PDB expression was slightly higher in 2-week pigs than 4-week pigs. In addition to these molecules associated with viral infection and intestinal homeostasis, also the intestinal microbiota influences the infection of enteric viruses. Therefore, the abundance of intestinal microbiota was analyzed by next-generation sequencing. This analysis showed that in the ileum of older pigs, and especially in 4 weeks pigs, higher levels of potential probiotics, like *Lactobacillus*, *Megasphaera*, *Prevetella_7*, *Mitsuokella*, *Dialister*, *Bifidobacterium* are present compared to in 1-week-old piglets (Fig. 3G and 3H). On the contrary, the ileum of 4-week-old piglets held lower numbers of pathogenic bacteria, like *Escherichia Shigella*, *Streptococcus*, *Weissella*, *Veillonella*, *Aeroccus*, and *Enterococcus*, compared to the younger piglets (Fig. 3I and 3J). In short, these results illustrate that in 1 week-old piglets, expression levels of viral invasion-assisting epithelial molecules were lower while expressions level of cytokines associated with intestinal health were higher. Furthermore, in 4-week-old pigs, more probiotic and less pathogenic microbiota were observed.

3.4. Higher expression levels of innate immune related genes in 4-week pigs

Next, the development of the innate intestinal immune system was investigated. Innate immunity, and especially type I IFN production was reported to play an important role in the defense against viruses. Therefore, expression level of IFNs and upstream TLR receptors were detected by RT-qPCR. In the duodenum and jejunum of 4-week-old pigs, higher expression levels of IFN-β were observed than in 1-week and 2-week-old pigs (Fig. 4A and 4B). In the ileum and MLN of 4-week-old pigs, higher levels of *IFN-β, TLR2, TLR3, TLR4, TLR9* were observed compared to the other age groups (Fig. 4C and 4D). In addition, expression levels of inflammatory cytokines in different intestinal segments as well as MLN were also analyzed by RT-qPCR. The Heat Map result illustrates that higher expression levels of inflammatory cytokines in 4-week-old pigs, especially in the ileum and MLN (Fig. 4E) compared to the younger pigs.

3.5. Better development of the adaptive immune system in 4-week pigs

Since the innate immunity in younger piglets is less well developed compared to older pigs, it is particularly important to determine whether adaptive immunity can provide a sufficient protection against exposure with enteric virus infection. Frequencies of B and T cells in blood and intestine were assessed by flowcytometry in pigs of different ages. Interestingly, these results (Fig. 5A, 5B, 5C) indicated that the PPs were full of immature B cells ($CD21^+$) (Sinkora et al., 2013), with lower percentages of $CD3^+$ T cells compared to PBMC and MLN. The percentage of $CD3^+$ T cells was higher in MLN than in PBMC. Moreover, in PBMC, the percentage of immature B cells ($CD21^+$) was highest in 4-week pigs (19.16 ± 5.902) (Fig. 5A) compared to the other two age groups (7.173 ± 0.6656 in 1-week, and 6.203 ± 0.4161 in 2-week). In PPs, the percentage of immature B cells was slightly decreased in 4-week pigs (86.733 ± 1.484) compared to 2-week pigs (91.100 ± 0.635) (Fig. 5B). While the percentage of T cells increased in 4-week pigs compared to 2-week pigs. In MLN, the percentage of immature B cells increased slightly with age and highest levels was observed in 4-week pigs (23.967 ± 5.695 in 1-week, 27.800 ± 0.814 in 2-week, 31.833 ± 4.901in 4-week). The percentage of T cells was observed highest in 1-week pigs (Fig. 5C). In conclusion, these data revealed that the percentage of immature B cells increased between 1-week. 2-week and 4-week pigs. This was especially observed in PP where immature B cells were hardly observed in piglets at 1-week of age.

To further understand B or T cell development in pigs of different ages, levels of cytokines that are known to play a role in lymphocyte differentiation were detected by RT-qPCR in different intestinal segments and MLN. These cytokine expression levels were higher in older pigs, especially in the ileum and MLN. In the duodenum, higher expression levels of *IL-6, IL-16*, and *IL-21* were observed in the

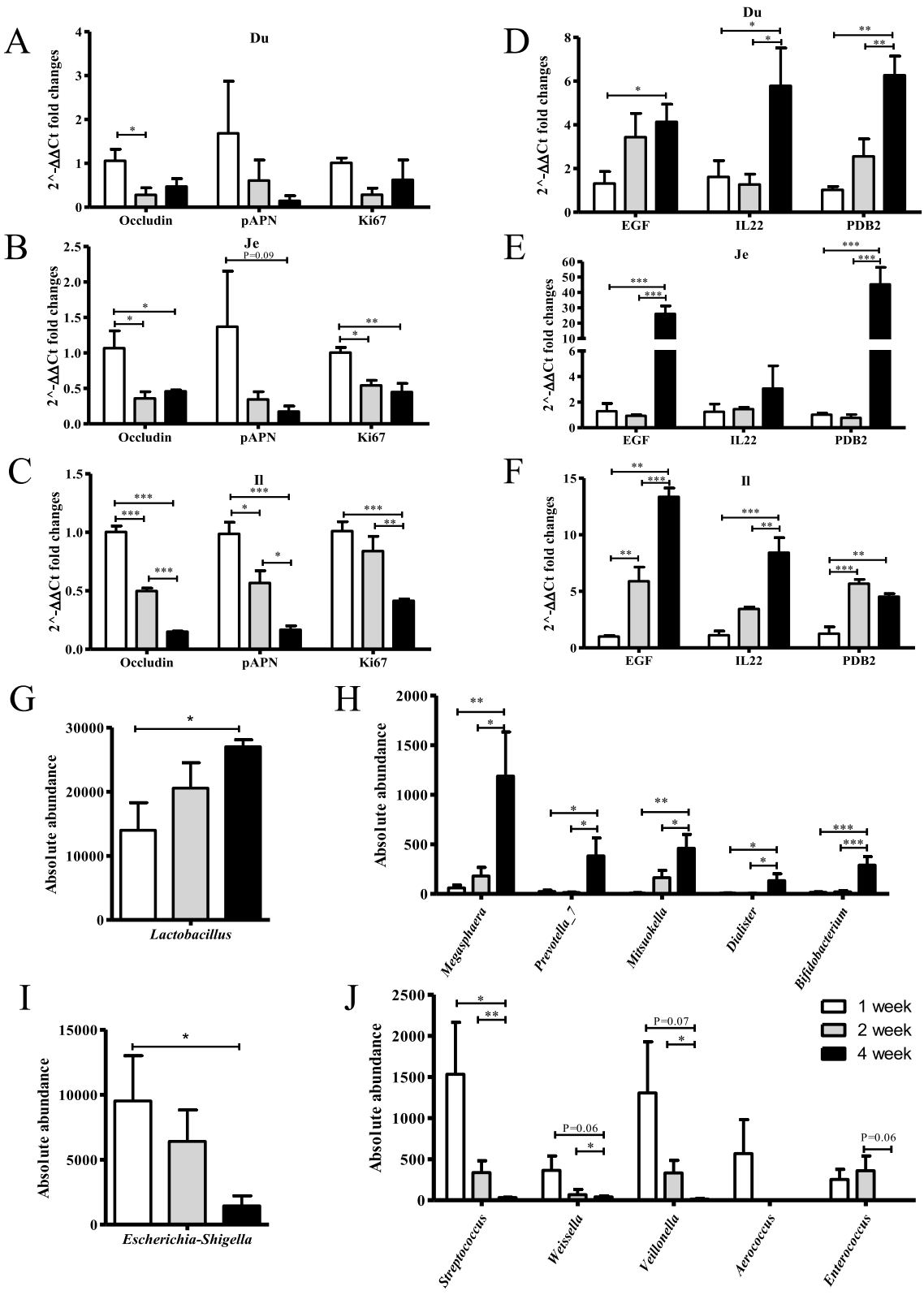

Fig. 3. Higher amount of enteric virus invasion-assisting elements in 1-week piglets. Real-time PCR was used to detect mRNA expression levels of *pAPN, occludin, Ki67, EGF, IL-22* and *pBD2* in duodenum, jejunum, and ileum (A–F). Numbers (Abundance?) of several commensal probiotic bacteria and pathogenic bacteria are pictured (G–J). Significant differences are indicated by ***$p < 0.001$, **$p < 0.01$, and * $p < 0.05$.

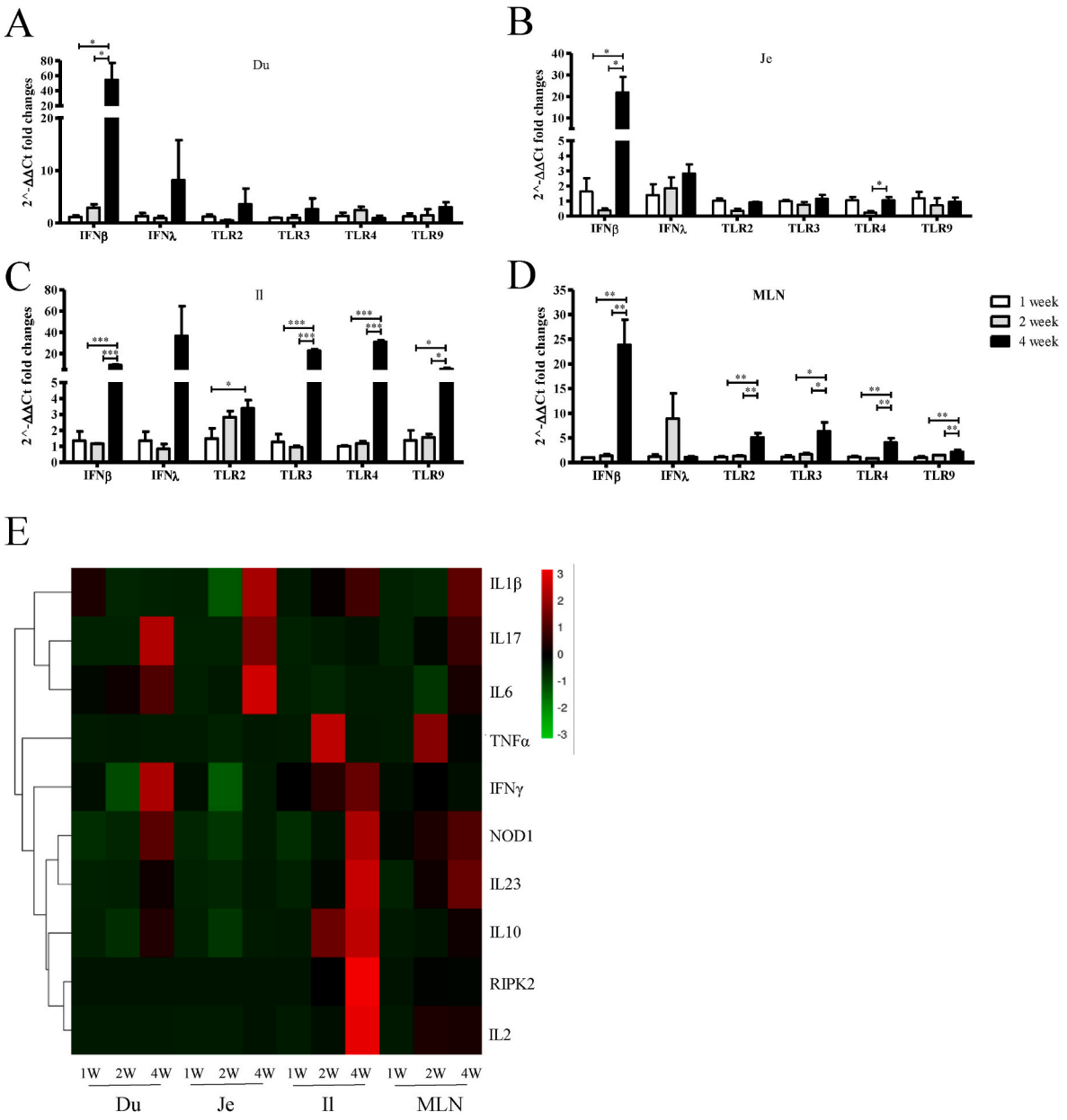

Fig. 4. Higher expression levels of innate immune related genes in 4-week pigs. Real-time PCR was used to detect mRNA expression level of IFNs and TLRs in duodenum (A), jejunum (B), and ileum (C) and MLN (D). Real-time PCR was used to detect mRNA expression levels of inflammatory cytokines in duodenum, jejunum, ileum and MLN, shown as a heatmap (E). Green means lowest expression level, red means highest expression levelst. Significant differences are indicated by ***$p < 0.001$, **$p < 0.01$, and * $p < 0.05$.

duodenum of 4-week-old pigs compared to 1-week-old piglets (Fig. 5D). In the jejunum, expression levels of IL-2, IL-6, and IL-16 were also highest in 4-week-old pigs (Fig. 5E). In the ileum, higher expression levels of *IL-7* and *IL-21* were observed in 4-week-old pigs (Fig. 5F). In the MLN, higher expression levels of all cytokines that were analyzed were observed in 4-week-old pigs, compared to 1-week-old pigs (Fig. 5G). Overall, highest expression levels of these immune-related factors were found in 4-week-old pigs. In combination with the increase in the percentage of B cells in pigs at four weeks of age comparing to younger piglets, we speculate that the adaptive immune system was better developed and more able to respond to a viral challenge.

4. Discussion

In this study, we show that the higher susceptibility of 1-week-old piglets for infection with pathogenic enteric viruses can be caused by the physiological structure of the villi, the composition of the intestinal microbiota and the difference in development of the intestinal immune system in comparison to older pigs. The schematic diagram shown in Fig. 6 summarizes possible reasons that may explain the high susceptibility of younger piglets for enteric virus infection. To sum up, younger piglets have higher levels of infection facilitating and assisting factors, while at the same time less inhibitory and protective factors are found as compared to older pigs.

First, when the intestine is exposed to virus, goblet cells may express higher levels of mucin2. This may lead to a more dense mucus layer in older pigs which can serve as a first line of defense (Kim and Khan, 2013). This thicker mucus layer can slow down the pace of viral infection or even remove the virus by trapping it into the MUC2 network (Hansson, 2012). Besides, in this dense mucus, β-defensin, type I antiviral IFNs and some inflammatory cytokines are also highly secreted. Also, these factors can inhibit virus infection and thereby maintain a

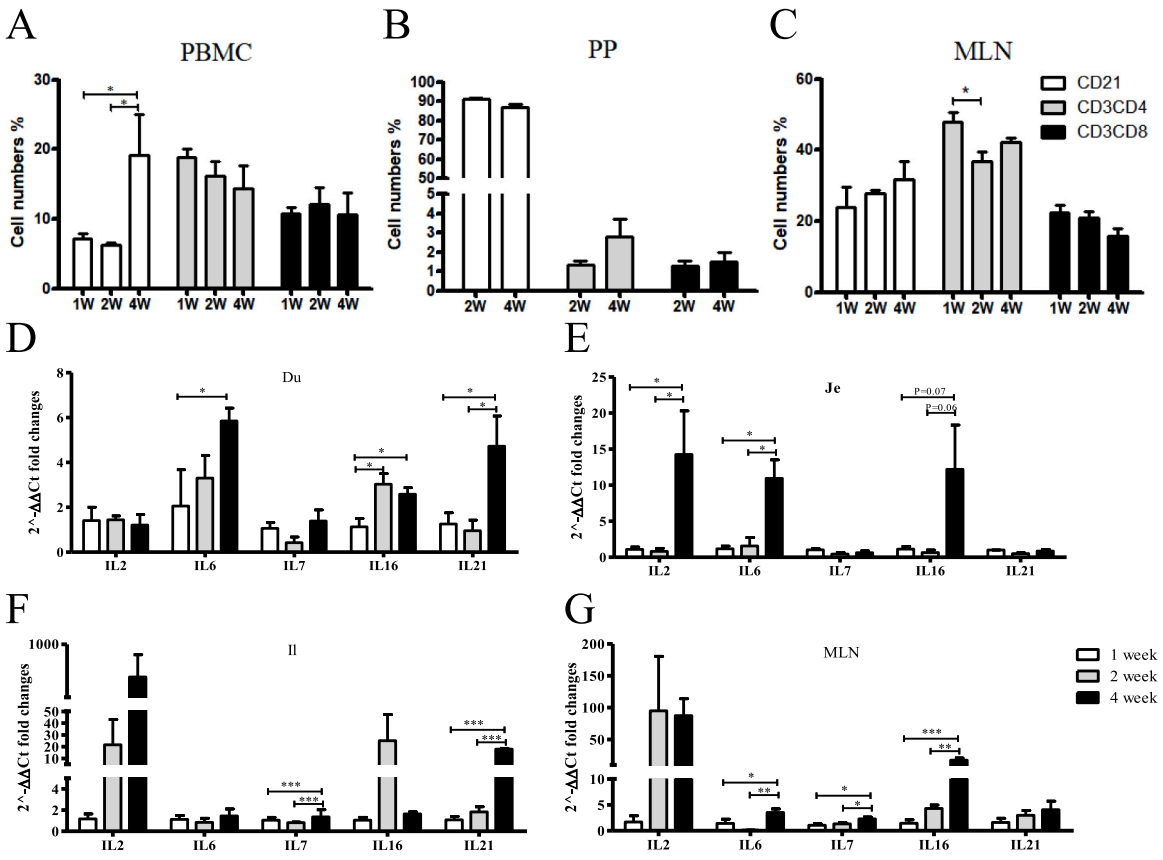

Fig. 5. Better development of the adaptive immune system in 4-week pigs. Flow cytometry was used to detect percetnages of $CD21^+$ immature B cells, $CD3^+CD4^+$ T cells and $CD3^+CD8^+$ T cells in PBMCs (A), PPs (B) and MLN (C). Total lymphocytes were gated based on size and complexity. Gating either of $CD21^+$, $CD3^+$, $CD4^+$, or $CD8^+$ lymphocytes using fluorochrome-coupled specific antibodies. Real-time PCR was used to detect mRNA expression levels of IL-2, IL-6, IL-7, IL-16, IL-21 in duodenum (D), jejunum (E), and ileum (F) and MLN (G). Significant differences are indicated by $^{***}p < 0.001$, $^{**}p < 0.01$, and $^{*}p < 0.05$.

healthy local microenvironment. More commensal and presumed probiotic bacteria, less pathogenic bacteria also modulate the balance of the intestinal microbiota, which may be beneficial for intestinal health (Walter, 2008). Hence, compared to the older pigs, 1-week piglets without these protective mechanisms, will be more susceptible to virus infection. Moreover, when viruses pass through the mucus layer and reach the surface of intestinal epithelial cells, the longer intestinal villi of piglets will be an easier target for attachment of the virus which will facilitate virus infection (Thomas et al., 2015). Higher expression levels of pAPN and occludin in epithelial cells of younger piglets can even further facilitate viral infection in the intestine (Liu et al., 2015b; Luo et al., 2017).

After viral infection of the intestinal epithelium, antigens will be captured and phagocytosed by APC that will present peptides derived from these antigens to the specific T cells in the lymph nodes, thereby initiating an immune response. The ileum of newborn piglets contains hardly any PPs. In addition, the low expression levels of IFNs and TLRs in contrast to that in older pigs indicates a less effective innate immune response in younger piglets, which is consistent with other studies (Nowacki et al., 1993). Therefore, young piglets display a structurally and functionally immature immune reactivity. On the contrary, older pigs not only have a better innate immune system but also a functional adaptive immune system. Because in pigs, the ileum consisted of continuous PPs and this is also the micro-environment for B cell maturation (Sinkora et al., 2011; Reboldi and Cyster, 2016), we evaluated the development of immune cells and the expression of the cytokines that drive this maturation. I observed increased expression levels of *IL-2, IL-6, IL-16, IL-21 and IL-7* may indicate enhanced differentiation and survival of T cells and B cells in older pigs. When the enteric virus infects older pigs, also the adaptive immune system can be stimulated, thereby generating high levels of anti-viral cytokines and specific antibodies to fight the virus. Lastly, if the epithelial layer is destroyed by direct interaction with the viruses (Liu and Wang, 2021), older pigs would show and a higher ability to repair the tissue. High expression levels of expressed *EGF* and *IL-22* can contribute to the repair of epithelial damage (Wang et al., 2019b; Hou et al., 2018). On the contrary, the longer intestinal villi in 1-week piglets will recover less well upon destruction by such a virus infection (Tivey and Smith, 1989).

5. Conclusion

In conclusion, by comparing structure and function of intestinal tissue, expression of molecules related to enteric viral infection, and the development of the immune system t in pigs of different ages, we found that younger piglets have more virus-facilitating elements and fewer virus-inhibitory factors compared to older pigs. This not only provides the basis for enteric virus-related disease susceptibility, but also provides a further direction for preventing and controlling diarrhea, particularly in young piglets.

Authors' contributions

GL and SY conceived the project. SY, NY and XH performed the experiments. SY, NY, GL, and YL analyzed the data. SY drafted the manuscript. GL, CJ, HS and NY edited the manuscript.

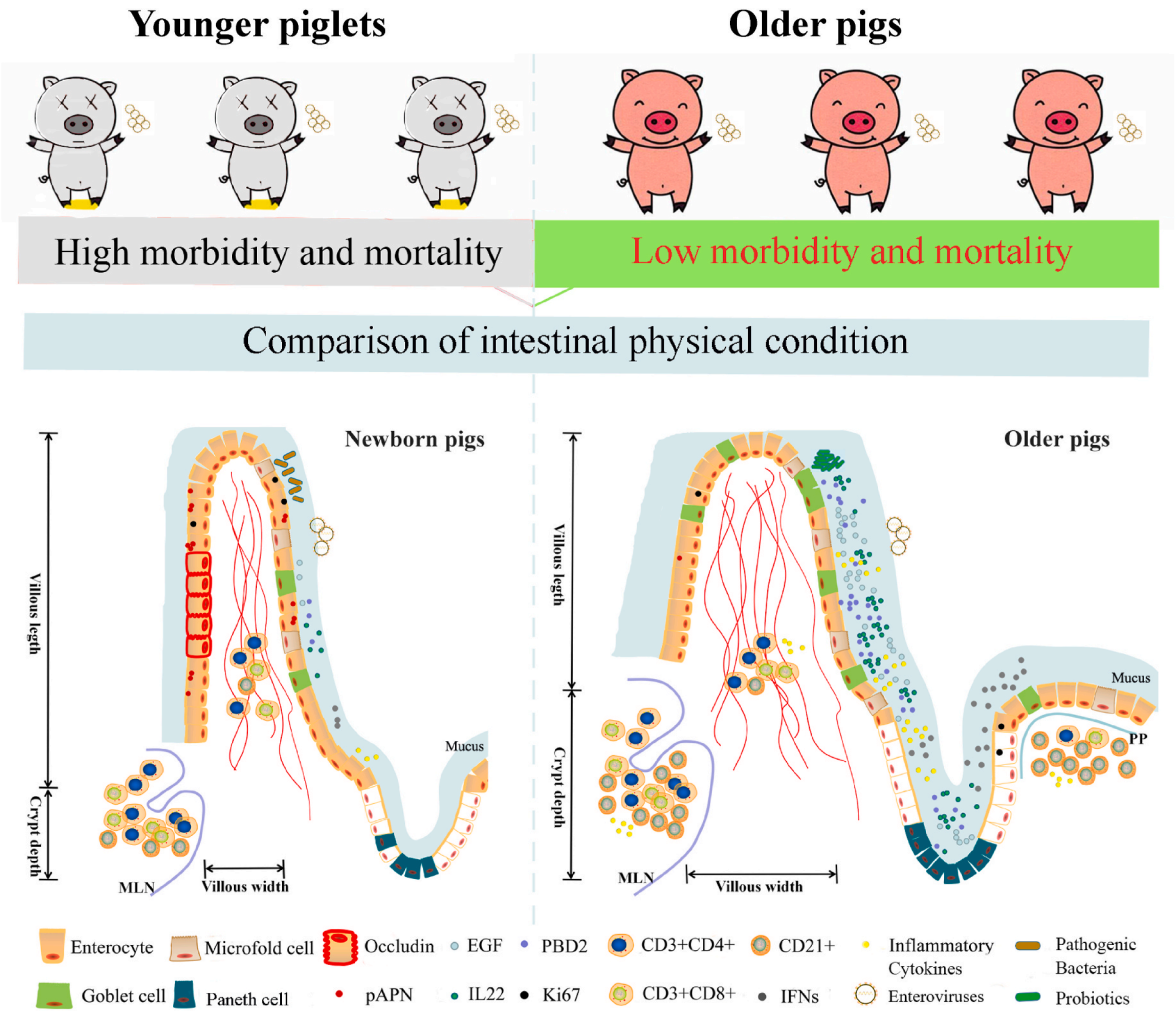

Fig. 6. A summarizing comparison of the observed differences between 1-week-old piglets (left) and older pigs (right).

Declaration of competing interest

The authors declare no conflicts of interest.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.dci.2022.104512.

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