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Developmental expression patterns and correlation analysis of *TLR4* and its downstream genes in the intestinal and immune tissues of Meishan pigs

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ABSTRACT - To understand the developmental expression patterns of key genes in the Toll-like receptor 4 (*TLR4*) pathway and their regulatory characteristics in the immune response in pigs, we examined *TLR4* and its downstream genes expression levels in the intestinal and immune tissues of Meishan pigs. The genes were expressed in all examined tissues at the different developmental stages. *TLR4* expression was higher in spleen and lower in other tissues. Spleen and lymph *TLR4* expression was significantly lower in 7- and 35-day-old pigs; in the intestinal tissues, it was significantly lower in 21- and 35-day-old pigs. *IFNA*, *IL1B*, and *TNFA* expression varied greatly with developmental stage; expression was significantly higher in most tissues in 21-, 134-, and 158-day-old pigs. *TLR4* was highly positively correlated with *TNFA* in the immune tissues and was significantly correlated with all downstream genes in the spleen; there was no significant correlation in the intestinal tissues. There was near significant positive correlation among the downstream genes in the intestinal tissues, but almost no significant correlation in the immune tissues. We speculated that the *TLR4* pathway genes may have an anti-lipopolysaccharide invasion effect during weaning, and the high expression of the downstream genes is beneficial for improving immunity in adult pigs. Our results may contribute to better understanding the *TLR4* signaling pathway and its molecular mechanisms and could provide a reference and basis for appropriate-age experimental animal selection for relevant future research.

Keywords: gene expression, growth curve, pig

Introduction

Upon entering the body, bacteria and viruses invade the innate immune system and induce an immune response. As important receptors involved in the innate immune system, Toll-like receptors (TLR) can specifically recognize pathogen-associated molecular patterns (PAMP), induce innate immunity, and then promote the acquired immunity (Lee and Min, 2007). *TLR4* plays a major role in cell inflammation and in the immune response. Many PAMP can bind specifically to TLR, and many types of PAMP can stimulate *TLR4* (Kurt-Jones et al., 2000; Rassa et al., 2002). As a principal component of the outer membrane of gram-negative bacteria, lipopolysaccharide (LPS) can specifically activate *TLR4* (Beutler et al., 2001), triggering a signal cascade that leads to the release of interferon alpha (*IFNA*), interleukin-1 beta (*IL1B*), tumour necrosis factor alpha (*TNFA*), and other downstream cytokines, which leads to intestinal inflammation (Plociennikowska et al., 2015). TLR signaling pathways play an important regulatory role in autoimmune diseases (Hamerman et al., 2016), viral infection (Abe et al.,

2007), and inflammatory bowel disease (Abreu et al., 2005). Our recent transcriptome sequencing results have indicated that the *TLR4* signaling pathway plays an important regulatory role in resistance to *Escherichia coli* F18 (Wu et al., 2016). The *TLR4* signaling pathway is a complicated protein interaction network, and whether the reaction is different in the distinct developmental stages of pigs remains unclear. Furthermore, the changes in *TLR4* signaling pathway gene expression levels during immune system development and before and after weaning remain unclear.

To reveal the developmental expression patterns of *TLR4* and its downstream genes, we detected the expression of the *TLR4* gene and its downstream genes *IFNA*, *IL1B*, and *TNFA* in the intestinal and immune tissues of Meishan pigs at various developmental stages. The results revealed the differential expression of this important immune signaling pathway at different developmental stages in pigs, and contributed to better understanding of the immune developmental changes during pig growth. Our findings can also provide a theoretical and experimental basis for studying the function and regulatory mechanism of the porcine *TLR4* signaling pathway.

Material and Methods

The local Institutional Animal Care and Use Committee approved the animal study proposal (case number: SYXK [Su] IACUC 2012-0029). All experimental procedures involving piglets were performed in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals and were approved by the State Council of the People's Republic of China.

We used Meishan pigs obtained from a company in Jiangsu, China. All experimental pigs were maintained under standard piggery conditions. The pigs had *ad libitum* access to a commercial-type compound feed containing 21.7% of crude protein and no antimicrobial additives or organic acids. We selected one pig from five litters at the various developmental stages (newborn, 7-, 14-, 21-, 28-, 35-, 134-, 158-day-old). A total of 40 pigs (five pigs per group) were used, and pigs at the same developmental stage were healthy and had similar characteristics (e.g., size and weight) (Table 1). The pigs were electrically stunned (300 V for 5 s) and bled by heart puncture under the left armpit. Spleen, thymus, lymph, duodenum, jejunum, and ileum tissue samples were obtained within 30 min after slaughter, frozen in liquid nitrogen, and stored at −80 °C.

Total RNA was extracted from the tissues (50-100 mg) using TRIzol (TaKaRa Biotechnology Dalian Co., Ltd.). The precipitated RNA was suspended in 20 μL RNase-free water, and stored at −80 °C. Quality of RNA was assessed by 1.5% formaldehyde denaturing gel electrophoresis. Concentration and purity of RNA were determined using a spectrophotometer (Nanodrop ND1000, NanoDrop Technologies Co., Ltd.).

Total RNA was reverse-transcribed into complementary DNA (cDNA) using a HiScript Q RT SuperMix kit for quantitative PCR (qPCR) (+genomic DNA [gDNA] wiper) (Vazyme Biotech Co., Ltd.). The cDNA synthesis reaction mixture (10 μL) consisted of 2 μL 5× qRT SuperMix II, 500 ng total RNA, and RNase-free water. The reaction was carried out at 25 °C for 10 min, 50 °C for 5 min, and 85 °C for 5 min, and the products were stored at 4 °C.

Based on published GenBank sequences, the primers for *TLR4* (NM_001113039.2), *IFNA* (NM_214393.1), *IL1B* (NM_001005149), and *TNFA* (NM_214022.1) were designed using Primer Premier 5.0 software (PREMIER Biosoft International, USA). The primers were synthesized by Takara Biotechnology Dalian Co., Ltd. *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) and *ACTB* (β-actin) were used as internal control genes to normalize the threshold cycle (Ct) values of the other transcripts. Table 2 lists the primer sequences and product lengths.

Real-time PCR amplification was performed using a PCR kit (Vazyme Biotech Co., Ltd.) in a 25-μL reaction mixture containing 2 μL cDNA, 0.5 μL forward and reverse primer (10 μM each), 0.5 μL 50 × ROX Reference Dye II, 10 μL 2× SYBR Green Real-Time PCR Master Mix, and double-distilled water. We used 7500 Real-Time PCR system (Applied Biosystems) to perform the process; PCR conditions were set at 95 °C for 5 min, followed by 40 cycles of 95 °C for 5 s and 60 °C for 34 s. Dissociation curve analysis

was performed after amplification. A peak melting temperature (Tm) of 60±0.8 °C in the dissociation curve was used to determine the specificity of the amplification product. The Tm value for each sample was calculated using the average of triplicate technical samples.

The original data were arranged using Excel 2007 software (Microsoft Corporation, USA). The relative quantitative expression results were calculated using the comparative Ct ($2^{-\Delta\Delta Ct}$) method (Livak and

Table 1 - Information of experimental pigs

Number	Days	Ear ID	Gender	Weight (kg)	Heart (g)
1	1	8738	Female	1.32	9.7
2	1	8877	Male	0.8	3.77
3	1	8766	Female	0.81	5.01
4	1	8891	Male	1.22	7.54
5	1	8903	Male	1.05	7.68
1	7	8736	Female	2.38	16.33
2	7	8881	Male	2.06	12.26
3	7	8772	Female	1.19	11.37
4	7	8883	Male	1.81	11.2
5	7	8794	Female	1.42	10.39
1	14	8849	Male	3.42	20.8
2	14	8750	Female	2.72	16
3	14	8905	Male	3.92	21.5
4	14	8887	Male	2.993	19.92
5	14	8897	Male	3.072	19.31
1	21	8734	Female	5.57	31.96
2	21	8788	Female	3.98	23.05
3	21	8867	Male	4.1	19.44
4	21	8891	Male	3.44	18.36
5	21	8764	Female	4.71	30.5
1	28	8754	Female	6.28	27.9
2	28	8853	Male	5.265	32.6
3	28	8774	Female	6.505	35.5
4	28	8889	Male	5.43	34
5	28	8911	Male	5.535	35.2
1	35	8796	Female	7.545	44.62
2	35	8776	Female	7.985	38.68
3	35	8871	Female	8.89	48.41
4	35	8889	Male	7.47	47.43
5	35	8855	Male	7.88	43.48
1	134	8826	Female	37.2	195
2	134	8834	Female	35.2	154.38
3	134	8802	Female	39.8	158.98
4	134	8909	Male	29.2	157.88
5	134	8786	Female	35.8	144.75
1	158	8056	Female	67	310
2	158	411635	Female	75	355
3	158	411266	Male	64	235
4	158	411725	Female	70	355
5	158	411639	Male	64.5	285

Table 2- Real-time PCR primer sequences

Gene	Accession no.	Primer sequence (5′→3′)	Tm (°C)	Length (bp)
TLR4	NM_001113039.2	F: CAGATAAGCGAGGCCGTCATT R: TTGCAGCCCACAAAAAGCA	60	113
IFNA	NM_214393.1	F: CCTGGACCACAGAAGGGA R: TCTCATGCACCAGAGCCA	60	92
IL1B	NM_001005149	F: TGATTGTGGCAAAGGAGGA R: TTGGGTCATCATCACAGACG	60	63
TNFA	NM_214022.1	F: CGACTCAGTGCCGAGATCAA R: CCTGCCCAGATTCAGCAAAG	60	58
GAPDH	AF017079.1	F: ACATCATCCCTGCTTCTACTGG R: TCTCCTCCTGCTCCAATCCAG	60	187
ACTB	XM_00312428.3	F: TGGCGCCCAGCACGATGAAG R: GATGGAGGGGCCGGACTCGT	60	149

Schmittgen, 2001). The general univariate linear model was used to determine differences in transcript levels among the developmental stages, age (or tissue) was taken as the fixed factor, and expression level as the dependent variables. A three-dimensional map was drawn using Origin 9.0 software (Microcal Software Inc., USA), and a heatmap was drawn using HemI 1.0 (Deng et al., 2014) after log2 conversion using the mean of the expression levels (X=log2(expression data)). Correlation analysis was performed using SPSS 18.0 software (SPSS Inc., Chicago, IL, USA) on the gene expression levels from the different tissues, respectively. Statistical significance was set at $P<0.05$.

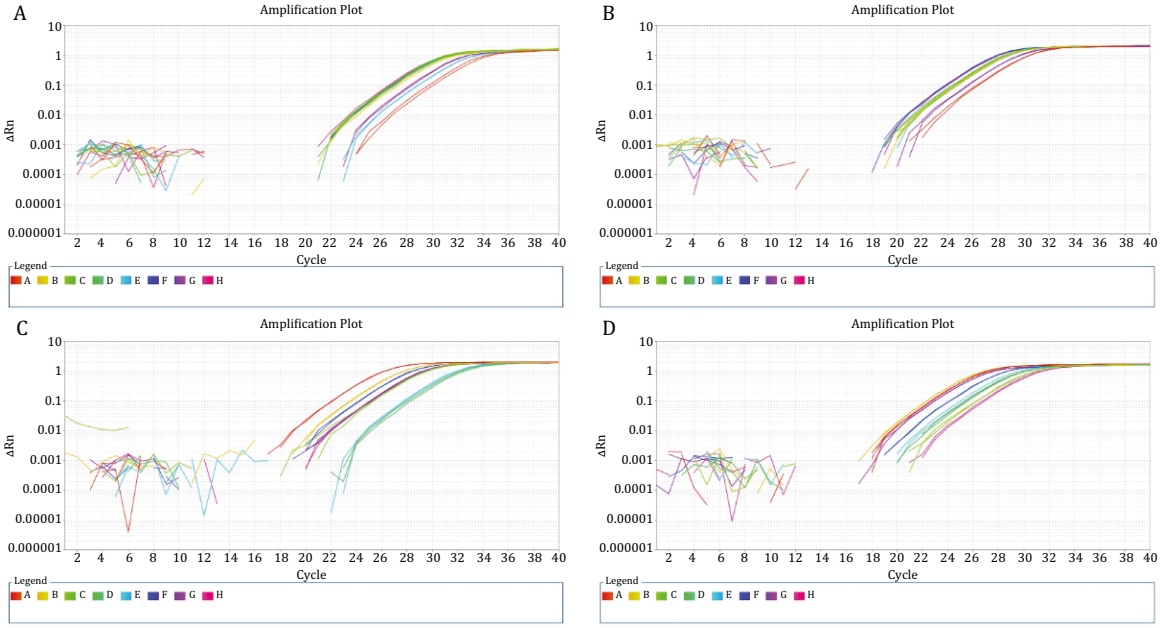
Results

There were three bands, i.e., 28S, 18S, and 5S, with no DNA contamination and obvious degradation, and the A260:A280 (absorbance at 260 nm and 280 nm) ratio was ~1.8–1.9. The results indicate that the extracted RNA had high integrity and purity and could be used in subsequent analysis.

The real-time PCR amplification and melting curves for the *TLR4*, *TNFA*, *IL1B*, and *IFNA* genes showed that the PCR product had only one definite peak, and no primer dimer or non-specific product (Figures 1 and 2).

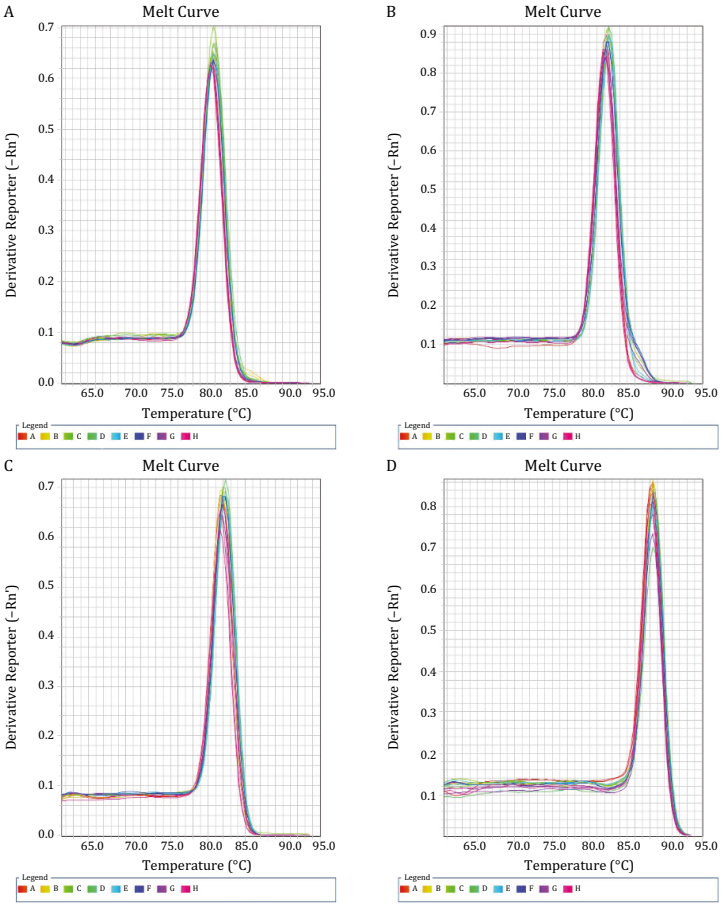
Expression levels of *TLR4*, *TNFA*, *IL1B*, and *IFNA* in the Meishan pig intestinal and immune tissues were detected by real-time PCR, and the results were normalized by the *GAPDH* and *ACTB* genes. *TLR4* and its downstream genes were expressed in all intestinal and immune tissues at the various developmental stages (Figure 3). *TLR4* expression was significantly higher in the spleen and significantly lower in the other tissues ($P<0.05$). *IFNA* expression was significantly higher in the small intestine (duodenum, jejunum, and ileum) and spleen ($P<0.05$), and lower in the other tissues. *IL1B* expression was significantly higher in the small intestine and lymph ($P<0.05$) and lower in the other tissues. *TNFA* expression was significantly higher in the thymus and lymph ($P<0.05$) and moderate in the other tissues.

TLR4 expression levels in the spleen and lymph were significantly lower at 7 and 35 days than at the other stages ($P<0.05$), and were significantly lower at 21-35 days in the intestinal tissues than at the other stages ($P<0.05$). Expression levels of *IFNA*, *IL1B*, and *TNFA* varied greatly with developmental stage in the examined tissues. *IFNA* expression levels were significantly higher at 21, 134, and 158 days



A, B, C, and D represent *TLR4*, *IFNA*, *IL1B*, and *TNFA* genes, respectively.
In the PCR process, the curve was taken as the abscissa and the real-time fluorescence intensity in the reaction process was taken as the ordinate.

Figure 1 - Real-time PCR amplification curves of *TLR4* and its downstream genes.



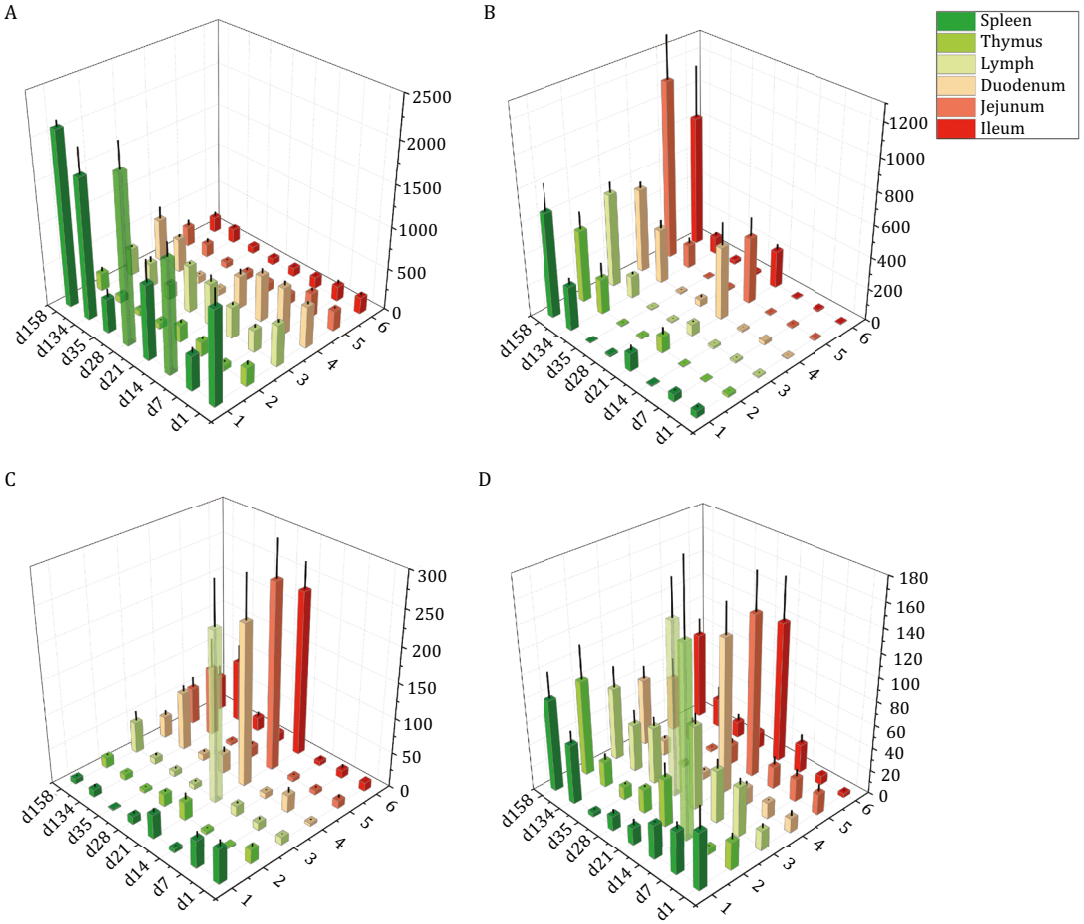
A, B, C, and D represent *TLR4*, *IFNA*, *IL1B*, and *TNFA* genes, respectively.
When the PCR product is heated, as the temperature increases, the double-stranded amplification product gradually melts, resulting in a decrease in fluorescence intensity. When reaching a certain temperature, a large amount of product is melted and the fluorescence is drastically decreased. By using this feature and the difference in Tm values of different PCR products, the temperature at which the fluorescent signal rapidly decreases is also different, and the specificity of PCR can be identified by this.

Figure 2 - Real-time PCR melting curves of *TLR4* and its downstream genes.

than at the other stages in all examined tissues (P<0.05). *IL1B* expression levels were significantly higher at 21 days than at the other stages in all examined tissues (P<0.05) and significantly higher at 134 and 158 days than at the other stages in the intestinal tissues (P<0.05). *TNFA* expression levels were significantly higher at 21 and 158 days than at the other stages in almost examined tissues (P<0.05), in addition to lower expression in spleen and thymus at these two stages.

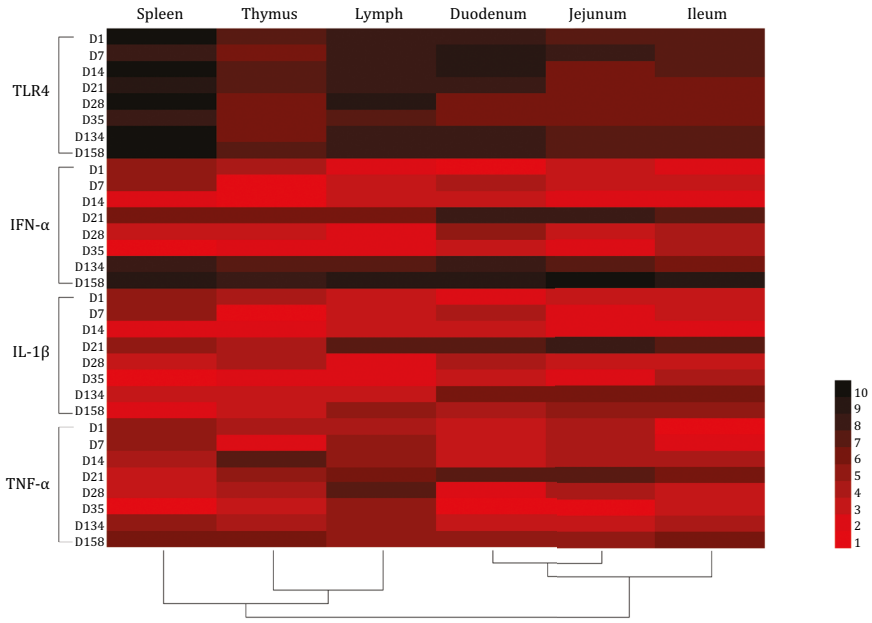
The intestinal and immune tissues could be clearly distinguished on the heatmap constructed based on the expression levels of the four genes (Figure 4). The thymus and lymph tissues were clustered together, followed by the spleen tissue. For the intestinal tissue, the duodenum and jejunum were first grouped together and then clustered with the ileum.

TLR4 and *IFNA* were significantly positively correlated in the spleen (R = 0.523, P<0.01) (Table 3). *TLR4* and *IL1B* were significantly negatively correlated in the spleen and thymus (R = −0.359, P<0.05; R = 0.524, P<0.01). *TLR4* and *TNFA* had significant positive correlations in the immune tissues (spleen, thymus, lymph: R = 0.400, P<0.05; R = 0.387, P<0.05; R = 0.452, P<0.01, respectively). There were significant positive correlations between *IFNA* and *IL1B* in the duodenum and jejunum (R = 0.693, P<0.01; R = 0.355, P<0.05, respectively); *IFNA* and *TNFA* had significant positive correlations in the spleen and intestinal tissues (duodenum, jejunum, ileum: R = 0.734, 0.720, 0.427, 0.549, respectively; P<0.01). *IL1B* and *TNFA* had significant positive correlations in the intestinal tissues (duodenum, jejunum, ileum: R = 0.847, 0.838, 0.811; P<0.01).



1 to 6 on the x-axis represent the spleen, thymus, lymph, duodenum, jejunum, and ileum, respectively.
The y-axis represents the eight developmental stages, and the z-axis represents relative expression levels of genes.
A, B, C, and D represent *TLR4*, *IFNA*, *IL1B*, and *TNFA* genes, respectively.
The lines in the bars denote the standard deviation.

Figure 3 - Spatiotemporal expression patterns of *TLR4* and its downstream genes in immunity and intestinal tissues from Meishan pigs.



The relative expression level was log2 transformed; then, the inputted expression data was linearly normalized, average linkage clustering for the hierarchical clustering.
Bar item number was set as 10, decimal places were set as 2.

Figure 4 - Heatmap of relative expressions of *TLR4* and its downstream genes.

Table 3 - Correlation analysis between *TLR4* and its downstream genes in Meishan pig intestinal and immune tissues

Tissue	Gene	*TLR4*	*IFNA*	*IL1B*	*TNFA*
Spleen	*TLR4*	1	0.523**	−0.359*	0.400*
	IFNA	0.523**	1	−0.216	0.734**
	IL1B	−0.359*	−0.216	1	0.157
	TNFA	0.400*	0.734**	0.157	1
Thymus	*TLR4*	1	0.283	0.524**	0.387*
	IFNA	0.283	1	0.149	0.090
	IL1B	0.524**	0.149	1	−0.143
	TNFA	0.387*	0.090	−0.143	1
Lymph	*TLR4*	1	−0.148	0.282	0.452**
	IFNA	−0.148	1	0.075	−0.003
	IL1B	0.282	0.075	1	0.089
	TNFA	0.452**	−0.003	0.089	1
Duodenum	*TLR4*	1	0.167	0.022	0.147
	IFNA	0.167	1	0.693**	0.720**
	IL1B	0.022	0.693**	1	0.847**
	TNFA	0.147	0.720**	0.847**	1
Jejunum	*TLR4*	1	0.239	−0.213	−0.113
	IFNA	0.239	1	0.355*	0.427**
	IL1B	−0.213	0.355*	1	0.838**
	TNFA	−0.113	0.427**	0.838**	1
Ileum	*TLR4*	1	0.256	−0.170	−0.148
	IFNA	0.256	1	0.261	0.549**
	IL1B	−0.170	0.261	1	0.811**
	TNFA	−0.148	0.549**	0.811**	1

* P<0.05; ** P<0.01.

Discussion

TLR4 is the first PAMP receptor to be characterized from the TLR family and comprises an extracellular domain, intracellular region, and a transmembrane domain. Its N-terminus contains multiple leucine-rich repeats that recognize PAMP, and the C-terminus contains a Toll/IL-1 receptor domain that recruits the downstream adaptor proteins to activate the downstream signaling pathways (Buchta and Bishop, 2014). The *TLR4* signaling pathway involves the innate immune responses triggered by most microorganisms, including gram-negative bacteria, *Chlamydia pneumoniae*, and some viruses, such as respiratory syncytial virus and mouse mammary tumor virus (Li et al., 2016). *TLR4* is a crucial LPS receptor: when LPS enters the bloodstream, it is bound by LPS-binding protein (LBP) in the serum and then transferred to CD14 (cluster of differentiation 14). CD14 is a high-affinity receptor of LPS and is present in secreted form or anchored to macrophage surfaces in the form of glycosylphosphatidylinositol, which decomposes LPS polymers into single molecules and presents them to *TLR4*/*MD-2* (lymphocyte antigen 96) complexes (Park and Lee, 2013; Gioannini et al., 2014). *TLR4* intracellular signaling is dependent on the MyD88 (myeloid differentiation primary response 88)-dependent and MyD88-independent pathways. Currently, there are numerous studies on the mechanism of the *TLR4* signaling pathway, and the relationship among the pathway genes is gradually becoming clear. However, knowledge of *TLR4* signaling pathway gene expression in all developmental stages of pigs remains relatively sparse. Therefore, we aimed to reveal the *TLR4*, *IFNA*, *IL1B*, and *TNFA* expression patterns at the various stages of development in Meishan pigs to provide important basic data for studying the function and mechanism of important genes in the *TLR4* signaling pathway in pigs, which can help deepen knowledge on the *TLR4* signaling pathway.

We found that *TLR4* and its downstream genes were expressed in all examined intestinal and immune tissues at the different developmental stages. Their expression levels in the various tissues differed, which may be due to the different functions of the tissues in response to pathogenic infections. *TLR4* expression was higher in the spleen and lower in the other tissues, which is in agreement with the study of Liu et al. (2016) on 35-day-old Meishan pigs. The spleen is the largest immune organ in the body, accounting for 25% of the total lymphoid tissue. It is the center of cellular and humoral immunity. This may explain why the spleen had higher *TLR4* expression than the other immune and intestinal tissues. The expression profiles of *IFNA*, *IL1B* and *TNFA* were found to be quite different in different tissues. Ojaniemi et al. (2003) found that, in mouse macrophages, LPS stimulation induces the formation of a complex between PI3K (phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha) and MyD88 and mediates the release of *IL1B* and *TNFA*. Furthermore, *IL1B* and *TNFA* secretion is linked to AKT activation of nuclear factor kappa B (NF-κB). In addition, the expression of *IFNA* does not only depend on the MyD88-dependent pathway (Honda and Taniguchi, 2006; Zhang and Lu, 2015). Therefore, we speculate that the expression profiles of these downstream genes may differ because they are not regulated by the same *TLR4* signal transduction pathway. The manner in which foreign pathogen invasion is addressed in the different developmental stages and organs of Meishan pigs may involve unique specifications regarding the expression of downstream genes in the *TLR4* signaling pathway, which are also worthy of further study.

In the present study, we analyzed the developmental expression patterns of *TLR4* and its downstream genes *IFNA*, *IL1B*, and *TNFA* in Meishan pigs. *TLR4* expression levels were highest in the spleen at the different developmental stages, which may be related to its important role in natural immunity, as it is the largest immune organ in pigs. *TLR4* is upstream of the *TLR4* signaling pathway, and its high expression level may be beneficial for initiating the immune response. The expression levels of the downstream genes *IFNA*, *IL1B*, and *TNFA* in the intestinal and immune tissues varied greatly with developmental stage. *IFNA* plays an important role in fighting viral pathogens (Capuron et al., 2002). As pro-inflammatory cytokines, *TNFA* and *IL1B* are the most important cytokines in the inflammatory reaction (Michaud et al., 2010; Fiorino et al., 2014). Activated inflammatory cells produce anti-inflammatory and pro-inflammatory cytokines, and the balance between the two helps control inflammation (Opal and DePalo, 2000; Gideon et al., 2015). The intestine is one of the main

target organs susceptible to pathogen invasion, and the immune tissues play a major role in local anti-infection function; therefore, we presume that the changes in *IFNA*, *IL1B*, and *TNFA* expression in the intestinal and immune tissues may be associated with this. The *IFNA*, *IL1B*, and *TNFA* expression levels at 21, 134, and 158 days were significantly higher than that of the other days in most of the examined tissues. Meishan pigs begin weaning when they are 21 days old. During this period, weaning is greatly stressful for piglets, and the changes in feed and living environment may lead to pathogenic diarrhea caused by *E. coli*. As *E. coli* is a Gram-negative bacteria, its adhesion leads to increased LPS concentrations in the intestine (Lallès et al., 2004; Fairbrother et al., 2005). Lipopolysaccharides can specifically activate the *TLR4* signaling pathway, ultimately leading to the release of downstream cytokines such as *IFNA*, *IL1B*, and *TNFA* (Plociennikowska et al., 2015); thus, we speculated that the increased expression levels of these genes in 21-day-old piglets may be associated with weaning stress and LPS infection. Meishan pigs attain sexual and physical maturity at 134 and 158 days, respectively. The high expression of the downstream genes of the *TLR4* signaling pathway in these two periods indicates that the regulatory mechanism of the inflammatory reaction in adult pigs may differ from that of piglets, i.e., it may be beneficial for enhancing immunity in adult pigs. The heatmap (Figure 4) shows that the expression patterns of the *TLR4* pathway genes in the intestinal and immune tissues could be clearly distinguished. After LPS and other foreign pathogenic products enter the body, the initial invasion site is the intestinal tract, and then LPS combines with *TLR4* expressed by the intestinal cells to attract inflammatory cells, and damages the mucosa (Liu et al., 2010). In turn, the immune organs such as the spleen, lymph, and thymus initiate the immune response when foreign pathogenic products such as LPS enter the circulatory system, and clear the LPS (Zhang et al., 1993; Norimatsu et al., 1995). In addition, *TLR4* signaling pathway stimulation can induce a potent inflammatory response. Accordingly, negative regulatory proteins such as NP105, ST2L, and SIGIRR are also expressed in the cell membrane to inhibit the *TLR4* signaling pathway, which is also essential for protecting the body from excessive inflammatory damage (Lu et al., 2008). These factors may be responsible for the differences in the expression patterns of the *TLR4* signaling pathway genes between the intestinal and immune tissues. The duodenum and jejunum had similar *TLR4* signaling pathway gene expression patterns, but that for the ileum differed from the two. Kamba et al. (2013) found that normal human intestinal epithelial cells only express small amounts of TLR4, while patients with ulcerative colitis and Crohn's disease overexpress *TLR4* in the epithelium of the colon and the end of ileum. In addition, the authors also found that *TLR4* is mainly distributed in the basal surface of the mucosal epithelium in ulcerative colitis patients, while it is concentrated at the top of the mucosal epithelium in Crohn's disease patients. Consequently, abnormal *TLR4* expression may lead to local immune abnormalities in the intestinal mucosa, wherein its sensitivity to different pathogens differs between intestinal segments. This may also be why the *TLR4* signaling pathway expression patterns of the ileum are distinct from that of the duodenum and jejunum.

The correlation analysis showed that *TLR4* and *TNFA* had significant positive correlations in the immune tissue; it is worth noting that there was significant correlation between *TLR4* and all of its downstream genes in the spleen, and there was some correlation in the other immune tissues, but no significant correlation in the intestinal tissues. This may be because intestinal immunity is mainly composed of gut-associated lymphoid tissue, immune cells, and immunologically active substances, such as secretory immunoglobulin A (SIgA), and forms the most important and complex part of the immune system (Walker, 2002). Of the internal organs, the intestinal tract comes into the closest contact with the exterior of the body. Nutrient absorption, development of a variety of biochemical reactions, symbiosis of various bacteria, and invasion of different antigens take place in the intestinal tract. The intestine also exerts autoimmune and synergistic effects with other immune tissues. In addition, other TLR can also combine with their respective ligands and activate a series of downstream factors, including NF-κB and mitogen-activated protein kinase (MAPK), to promote the production of inflammatory cytokines and other factors (Serezani et al., 2011). There was a near significant positive correlation among the downstream genes in the intestinal tissues, but no significant correlation among the immune tissues (except for *IFNA* and *TNFA* in the spleen). *IFNA*, *IL1B*, and *TNFA* are closely related to the inflammatory response caused by various intestinal

diseases. Based on their diverse roles in immune regulation, cytokines can be divided into pro-inflammatory and anti-inflammatory cytokines. The imbalance between cytokines is one of the principal factors of mucosal damage (Dinarello, 2000; Opal and DePalo, 2000). *IFNA* has a dual role of antiviral and immune regulation; *IL1B* and *TNFA* are important pro-inflammatory cytokines. Under normal physiological conditions, these cytokines maintain a balance to protect the integrity of the intestinal mucosa. This may be why these downstream genes were significantly positively correlated in the intestinal tissues of the Meishan pigs throughout their development.

Conclusions

In this study, we found that *TLR4* signaling pathway, as an important immune regulatory pathway in pigs, showed an interesting pattern of change. We speculated that *TLR4* pathway genes may have an anti-lipopolysaccharide invasion effect during weaning and that the high expression of the downstream genes is beneficial for improving immunity in adult pigs. We systematically demonstrated that the mRNA expression changes of *TLR4* signaling pathway during the immune development of pigs provided experimental and theoretical basis for the research of *TLR4* signaling pathway.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Conceptualization: W. Bao and S. Wu. Data curation: W. Qin and Y. Cao. Formal analysis: W. Qin and Z. Gao. Methodology: W. Qin, Z. Gao, Y. Cao and Z. Wu. Software: W. Qin and Z. Gao. Validation: Y. Cao. Writing-original draft: W. Qin, Z. Gao, Y. Cao and Z. Wu. Writing-review & editing: W. Bao and S. Wu.

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