Polish Journal of Veterinary Sciences Vol. 19, No. 2 (2016), 303–308

DOI 10.1515/pjvs-2016-0037

Original article

Differential expression of Toll-like receptor 4 signaling pathway genes in *Escherichia coli* F18-resistant and – sensitive Meishan piglets

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Abstract

The Toll-like receptor 4 (*TLR4*) signaling pathway is an important inflammatory pathways associated with the progression of numerous diseases. The aim of the present study was to investigate the relationship between *TLR4* signaling and resistance to *Escherichia coli* F18 in locally weaned Meishan piglets. Using a real-time PCR approach, expression profiles were determined for key *TLR4* signaling pathway genes *TLR4*, *MyD88*, *CD14*, *IFN-α*, *IL-1β* and *TNF-α* in the spleen, thymus, lymph nodes, duodenum and jejunum of *E. coli* F18-resistant and -sensitive animals. *TLR4* signaling pathway genes were expressed in all the immune organs and intestinal tissues, and the expression was generally higher in the spleen and lymph nodes. *TLR4* transcription was higher in the spleen of sensitive piglets ($p<0.05$), but there was no significant difference in *TLR4* mRNA levels in other tissues. Similarly, *CD14* transcription was higher in lymph nodes of sensitive animals ($p<0.05$) but not in other tissues. *IL-1β* expression was higher in the spleen and in the duodenum of resistant piglets ($p<0.05$, $p<0.01$, respectively), and there were no significant differences in other tissues. There were also no significant differences in the expression of *MyD88*, *TNF-α* and *IFN-α* between sensitive and resistant piglets ($p>0.05$). These results further confirm the involvement of the *TLR4* signaling pathway in resistance to *E. coli* F18 in Meishan weaned piglets. The resistance appeared to be mediated via downregulation of *TLR4* and *CD14*, and upregulation of *MyD88* that may promote the release of cytokines *TNF-α*, *IL-1β*, *IFN-α* and other inflammatory mediators which help to fight against *E. coli* F18 infection.

Key words: pig, TLR4, cytokine, immune response, *E. coli* F18

Introduction

Post-weaning diarrhea (PWD) in piglets is the most frequent acute and fatal disease affecting the swine industry at present, and the enterotoxigenic *Escherichia coli* (ETEC) F18 strain is the main pathogen causing the disease (Imberechts et al. 1992, Rippinger et al. 1995, Imberechts et al. 1997). The F18 fimbriae expressed by porcine toxigenic *E. coli* strains are comprised of 1-2 um long filaments that mediate adhesion to enterocytes and once adhered, bacteria enter the cell where they multiply and mature, releasing

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enterotoxins in the process (Hahn et al. 2000). While the use of antibiotics and vaccines can reduce diarrhea resulting from *E. coli* infections, long-term treatment results in increased antibiotic-resistant ETEC, and large numbers of pathogenic bacteria continue to reside both in piglets and the surrounding environment. It is therefore preferable to improve resistance to PWD through genetic approaches in order to solve the problem at source. Some research on ETEC F18 resistance has been reported; Vogeli et al. (1997) have argued that the *FUT1* G(M307)A point mutation is a useful marker for identifying *E. coli* F18 adhesion-resistant in pigs. However, multiple studies have indicated that this functional site is not usually suitable for Chinese indigenous pig breeds (Yan et al. 2003, Bao et al. 2008). Thus, screening for candidate genes that may be responsible for resistance to *E. coli* F18 in Chinese native pig breeds and identifying effective genetic markers remains a priority.

Mammalian Toll-like receptors (TLRs) derive their name from the Drosophila Toll protein, and TLRs are responsible for the activation of innate immunity, induction of the adaptive immune defense mechanism, and are associated with congenital innate immunity (Medzhitov et al. 2001, Beutler et al. 2005). Mammalian TLRs constitute a large family of transmembrane receptors, of which *TLR4* has received the most attention and has been found to induce the production of inflammatory factors and cytokines during anti-infection and immunity processes (Takeda and Akira 2005, Zacharowski et al. 2006, Pan et al. 2012). We previously used Agilent two-color microarray-based gene expression profiling to investigate differential gene expression, and found that the *TLR4* signaling pathway may regulate resistance to *E. coli* F18 in weaned piglets (Bao et al. 2012). Meishan pigs, a popular Chinese local breed, were infected with *E. coli* F18 and examination of intestinal tissue, bacterial counts and binding assays revealed two distinct resistant and sensitive phenotypes (Wu et al. 2014). The present study investigated the expression profiles of resistant and sensitive pigs, and assessed the differential expression of *TLR4* signaling pathway genes *TLR4*, *MyD88*, *CD14*, *IFN-α*, *IL-1β* and *TNF-α* in particular. We chose three distinct types of immune organs (spleen, thymus, lymph node) and two distinct types of intestinal tissues (duodenum and jejunum). These results provide a theoretical basis for screening potential resistance-associated genes and may help to identify genetic markers of *E. coli* F18 infection in pigs.

Materials and Methods

Experimental materials and sample collection

Meishan pigs (35 days old) were obtained from the Meishan Pig Conservation Breeding Company. Prior to the main study, we selected weaning piglets at 35 days of age with almost the same birth weight and weaning weight as model animals, those piglets were from four families and tested their susceptibility to *E. coli* F18 by challenging with the pathogens through feeding F18ab and F18ac strains (Wu et al. 2014), and then we obtained 27 piglets with diarrhea and 15 piglets without diarrhea. The intestinal tract of these pigs were used to carry out series of verification tests, such as *E. coli* F18 bacteria counting, histopathological detection and adhesion test of the pathogens to the epithelial cells of the small intestine in vitro (Liu et al. 2013). Finally we attained confirmed individuals with the resistance and susceptibility to *E. coli* F18, from which we selected two resistant and two sensitive full-sib piglets from four families respectively (in total eight resistant and eight sensitive piglets, eight full-sib pairs). The spleen (the central part of spleen), thymus (the whole thymus), mesenteric lymph nodes, duodenum (the forepart of duodenum) and jejunum (the forepart of jejunum) tissues were sampled from all the individuals. About 100 mg of the sample was placed in 1.5 mL nuclease-free Eppendorf tubes, frozen immediately in liquid nitrogen and stored at -80°C until further experimentation.

Real-time PCR primer design

Primers for real-time PCR amplification of *TLR4*, *MyD88*, *CD14*, *IFN-α*, *IL-1β* and *TNF-α* were designed based on gene sequences obtained from the GenBank database. *GAPDH, TBP1 and ACTB* were used as an internal control to normalize threshold cycle (C_t) values. Primers (Table 1) were synthesized by Shanghai Invitrogen Biotechnology (Shanghai, China).

Total RNA extraction and fluorescence quantitative PCR

Total RNA was extracted from the tissue samples (50-100 mg) using Trizol reagent (TaKaRa Biotechnology Dalian Co., Ltd, China) according to manufacturer's instructions. Qualitative and quantitative assessment of RNA was carried out by agarose gel

Table 1. Primers used for real-time PCR.

Gene	Accession No.	Sequence (5'→3')	Length (bp)
TLR4	AB232527	F: CAGATAAGCGAGGCCGTCATT R: TTGCAGCCCACAAAAAGCA	113
CD14	AB267810.1	F: CCTCAGACTCCGTAATGTG R: CCGGGATTGTCAGATAGG	180
MyD88	EF198416.1	F: GCTGGAACAGACCAACTAT R: TCCTTGCTTTGCAGGTAAT	153
TNFα	X54001	F: CGACTCAGTGCCGAGATCAA R: CCTGCCCAGATTCAGCAAAG	58
IL-1β	NM_001005149	F: TGATTGTGGCAAAGGAGGA R: TTGGGTCATCATCACAGACG	63
IFN-α	X57191	F: CCTGGACCACAGAAGGGA R: TCTCATGCACCAGAGCCA	92
GAPDH	AF017079.1	F: ACATCATCCCTGCTTCTACTGG R: CTCGGACGCCTGCTTCAC	187
TBP1	DQ845178.1	F:AACAGTTCAGTAGTTATGAGC R: AGATGTTCTCAAACGCTTCG	153
ACTB	XM_00312428.3	F:TGGCGCCCAGCACGATGAAG R:GATGGAGGGGCCGGACTCGT	149

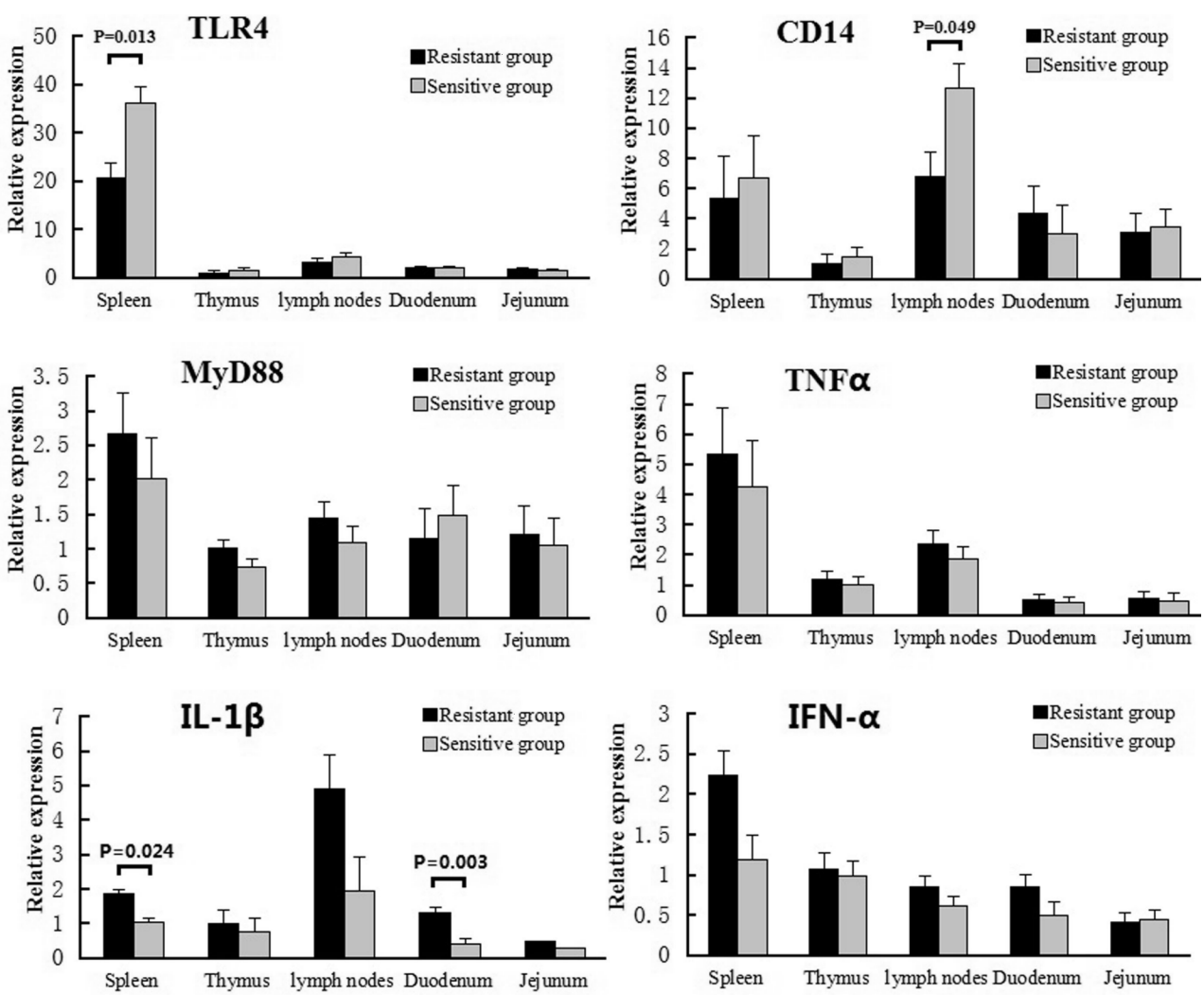

Fig. 1. Expression of *TLR4*, *MyD88*, *CD14*, *IFN-α*, *IL-1β* and *TNF-α* in different tissues of *E. coli* F18-sensitive and -resistant Meishan piglets. Samples were collected from the resistant group (n=8) and sensitive group (n=8) of piglets; three technical repetitions in qPCR analysis of every gene were performed.

electrophoresis and NanoDrop 1000 Spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USE), respectively. Precipitated RNA was dissolved in 20 μL of RNase-free H_2O and stored at -70°C.

cDNA was synthesized using a Reverse Transcription System (TaKaRa Biotechnology Dalian Co., Ltd, China) according to manufacturer's instructions. Briefly, reactions contained 2 μL of 5 × PrimeScript buffer, 0.5 μL of PrimeScript RT Enzyme Mix I, 0.5 μL of Oligo dT, 0.5 μL of random hexamers, 500 ng of total RNA, and RNase-free Water to a final volume of 10 μL. Reactions were carried out at 37 for 15 min and at 85 for 5 s.

Real-time PCR amplification was performed in 20 μL reactions containing 1 μL of cDNA (100-500 ng), 0.4 μL of forward and reverse primer (10 μL of each), 0.4 μL of 50 × ROX Reference Dye II, 10 μL of 2 × SYBR Green Real-time PCR Master Mix, and 6.8 μL of double-distilled H_2O. Thermal cycling was performed as follows: 95 for 15 s, followed by 40 cycles of 95 for 5 s and 62 for 34 s. The dissociation curve was analyzed, and a T_m peak at 85 ± 0.8 was used to determine the specificity of amplification. T_m values are averages of triplicate experiments.

Data processing and analysis

The $2^{-\Delta\Delta Ct}$ method, where ΔΔCt = (average Ct value of the target gene – average Ct value of the housekeeping gene) – (average Ct value of the control gene – average Ct value of the housekeeping gene), was used to process real-time PCR data (Livak et al. 2001). Statistical analysis was carried out using SPSS 16.0 software (SPSS Inc, USA), and the Student's t-test was applied to determine significant differences in mRNA expression between different animal groups.

Results

Using the established SYBR green real-time quantitative PCR method described above, the expression levels of *TLR4* signaling pathway genes (*TLR4*, *MyD88*, *CD14*, *IFN-α*, *IL-1β* and *TNF-α*) were examined in five tissues (spleen, thymus, lymph node, duodenum and jejunum) in *E. coli* F18-resistant and -susceptible individuals. The expression level of each gene in the thymus tissue of the resistant group was defined as 1. *TLR4* signaling pathway genes were expressed in all tissues tested, and expression was highest in the spleen and lymph nodes (Fig. 1). *TLR4* expression was higher in the spleen of sensitive piglets ($p<0.05$), but there was no significant difference in *TLR4* mRNA levels in other tissues. *CD14* transcription was also higher in lymph nodes in sensitive animals ($p<0.05$) but not in other tissues. *IL-1β* expression was higher in the spleen and in the duodenum of resistant pigs ($p<0.05$, $p<0.01$ respectively). No significant differences were found in the expression of *IL-1β* in other tissues, and no significant differences were detected in the expression of *MyD88*, *TNF-α* and *IFN-α* between sensitive and resistant piglets in any tissues ($p>0.05$).

Discussion

TLRs are cell membrane pattern recognition receptors expressed predominantly in cells that participate in the host defense against bacteria (Pasare and Medzhtov 2004). TLRs recognize and bind to highly conserved molecular structures present on or associated with particular pathogens, and in doing so trigger signal transduction cascades that lead to the release of inflammatory mediators. TLRs play an important role in the innate and acquired immune response systems. Innate immune responses caused by TLR4 stimulation are mediated through two signaling pathways, called the *MyD88*-dependent and *MyD88*-independent pathways, which utilize adaptor proteins MyD88/TIRAP and TRIF/TRAM, respectively. *TLR4* is the most widely-studied member of the TLR receptor family, and the *TLR4*/*CD14* signaling pathway is one of the most important pathways involved in mediating the inflammatory response to bacterial endotoxins. This receptor also influences the development of other diseases. The *TLR4* signaling pathway functions primarily through classical *MyD88*-dependent pathway activation. The subsequent downstream releases inflammatory cytokines, which in turn trigger the host immune response. The signal can also be conducted through *MyD88*-independent pathway. The *MyD88*-independent pathway can generate surprisingly robust IgG antibodies to play an role in antiviral effects (Yamamoto et al. 2003a,b, Rogers et al. 2015). Identification of drugs that can block or inhibit nodes in the *TLR4* signaling pathway are therefore of great clinical interest. A number of studies on *TLR4* signaling pathway genes in humans and mice have demonstrated an important role in immunity, disease and infection (Takeda et al. 2005, Zacharowski et al. 2006, Pan et al. 2012), and they may therefore be associated with resistance in other animals.

In the present study we investigated the expression key *TLR4* signaling pathway genes in the spleen, thymus, lymph nodes, duodenum and jejunum

of *E. coli* F18-resistant and -sensitive Meishan piglets as a representative Chinese breed. The expression was found to be higher in the spleen and lymph nodes, and since these both are important immune organs, the *TLR4* signaling pathway does appear to contribute to the host immune response in pigs. Differential expression profiling showed that *TLR4* mRNA levels were higher in the spleen of the sensitive individuals ($p<0.05$), while there was no significant difference in other tissues, although the expression of *TLR4* in the sensitive pigs was generally higher than that in resistant animals. This result may reflect the fact that *TLR4* is the main receptor that identifies lipopolysaccharide (LPS) and so mediates the inflammatory response against a range of pathogenic bacterial species. Indeed, we previously demonstrated that *TLR4* elicits the host innate immune recognition mechanism to Gram-negative bacteria including *E. coli* F18, and downregulation of *TLR4* expression may be related to resistance (Bao et al. 2011).

CD14 mRNA levels were significantly higher in the lymph nodes of the sensitive pigs ($p<0.05$), but there was no significant difference in other tissues, although as observed with *TLR4*, the expression of *CD14* was generally higher in sensitive animals CD14 may have a particularly high affinity for LPS, since they are known to be specific recognition sites for *LPS* (Peri et al. 2012). CD14 combines with LPS binding protein (LBP) and passes LPS on to TLR4 receptor complexes (Wright et al. 1990). Previous studies have shown that CD14-/- in mice are resistant to peritoneal infection with some clinical isolates of *E. coli* (Haziot et al. 2001). Thus the increased expression of *CD14* may increase the risk of infection (Metkar et al. 2012), and downregulation of *CD14* may therefore be associated with resistance to *E. coli* F18.

The expression of *MyD88*, *TNF-α*, *IL-1β* and *IFN-α* was generally higher in all the tissues in the resistant pigs, and levels of *IL-1β* mRNA in resistant spleen and duodenum tissue were particularly elevated ($p<0.05$; $p<0.01$). Myeloid differentiation factor 88 (MyD88) is an essential adaptor in the TLR-induced inflammatory response(Takeda et al. 2003). Following activation of the signal transduction cascade, the activation of the *MyD88*-dependent pathway leads to production of nuclear transcription factors (Pandey and Agrawal 2006), and activated NF-κB transmits the signal to the nucleus, resulting in increased transcription of *TNF-α* and *IL-1β*. This further activates NF-κB via a positive feedback mechanism that stimulates the release of cytokines IL-6 and IL-8, and interferons *IFN-α* and *IFN-β*, resulting in amplification of the initial inflammatory stimulus (Hawiger 2001). Whereas *MyD88*-independent pathway activation induces production of IFN-inducible cytokines such as RANTES and IFN-*β* (Hoebe et al. 2003). Transcription of many pro-inflammatory cytokine genes is regulated by the transcription factor NF-κB. The early activation of NF-κB mediated through TLR4 depends on the *MyD88*-dependent pathway but its late activation involves both *MyD88*-dependent and -independent pathways (Kawai et al. 1999, Hoebe et al. 2003). This signaling pathway therefore controls the expression of cytokines that activate the host immune system (Dauphinee and Karsan 2006, Liu et al. 2006). Together, these results and previous findings indicate that upregulation of *MyD88* in the resistant pigs leads to a series of inflammatory responses including increased release of cytokines such as *IL-1β* and other inflammatory mediators, which accelerate chemotaxis and aggregation of neutrophils and macrophages, increase capillary permeability, and assists infiltration of lymphocytes. All of these inflammatory responses may contribute to resistance to *E. coli* F18 infection.

The present results indicate that resistance to *E. coli* F18 in pigs is associated with downregulation of *TLR4* and *CD14*, and possible upregulation of *MyD88*. We intend to further investigate the regulatory functions of *TLR4*, *CD14*, *IL-1β* and other genes at the cellular level using RNAi and overexpression approaches. Resistance-associated *TLR4* signaling pathway SNPs will also be probed to identify genetic markers for the diagnosis and treatment of *E. coli* F18 infections in pigs and other animals.

Acknowledgments

This study was funded by the National Key Technology R&D Program of the Ministry of Science and Technology (2015BAD03B01), the Genetically Modified Organisms Technology Major Project (No. 2014ZX0800601B), Science and Technology Supporting Project of Jiangsu Province (BE2014357, BE2015329) and the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD).

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