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Identification of genes and pathways related to lipopolysaccharide signaling in duckling spleens

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ABSTRACT. Lipopolysaccharide (LPS), the major component of the outer cell wall of Gram-negative bacteria, activates the immune system and threatens the health of livestock and poultry. However, little is known about the genes and pathways involved in the immune response of ducklings to LPS. To elucidate the genes involved in the response of 7-day-old duckling spleens treated with LPS, RNA from LPS-treated and control duckling spleens was analyzed by RNA-Seq. The results showed 11,095 and 10,840 genes with >10 clean reads in the LPS-treated and control groups, respectively. Among these genes, 89 were differentially expressed ($\log_2$ ratio ≥ 1, P ≤ 0.01, false discovery rate ≤ 0.001); 67 of these were upregulated and 22 were downregulated in the LPS-treated group compared to the control. GO and GO-rich analysis showed that differentially expressed genes were enriched in 13 functional categories (P < 0.05). Pathway analysis and pathway richness analysis showed that differentially expressed genes were enriched in six pathway categories (P < 0.05). Further analysis showed that some immune system-related

signaling pathways, such as the hematopoietic cell lineage, Toll-like receptor signaling pathway, T cell receptor signaling pathway, T cell receptor signaling pathway, complement and coagulation cascades, antigen processing and presentation, and chemokine signaling pathway, are active during the immune response. To confirm the RNA-Seq results, we detected *CCL4*, *LBP*, *CD71*, and *STEAP3* expression using real-time PCR analysis, and the results were consistent with the RNA-Seq results. Our results provide new information on the genes involved in the immune response of duckling spleens to LPS.

Key words: Duck; Spleen; LPS; Differentially expressed genes

INTRODUCTION

Gram-negative bacteria (which appear red on Gram staining) can be detrimental to animal health in the livestock and poultry production industries. Lipopolysaccharide (LPS) is the major component of the outer cell wall of Gram-negative bacteria (Mani et al., 2012). Researchers have shown that a potent immunostimulant is responsible for much of the pathogenicity associated with Gram-negative bacterial infection (Leveque et al., 2003). When bacteria are killed by disinfectants, the presence of endotoxin (LPS) that remains in the animal building or in the air could damage the immune system and threaten the health of livestock and poultry (Pomorska et al., 2007).

LPS-induced damage has been studied in other species such as pig, rat, and chicken (Lipton et al., 2001; Parmentier et al., 2009; Chen et al., 2013); however, little information is known about the damage caused by LPS in ducks. Maloney and Gray (1998) showed that LPS could cause a temperature increase and prolonged fever in ducks. In animal production, the ducklings had a weaker defense and immune response to Gram-negative bacterial diseases compared with the adult ducks. Our research has also shown that the liver and spleen can be damaged by LPS in ducklings (unpublished data). Studies investigating the gene interactions involved in the immune response of duckling spleens to LPS will be useful for researchers and duck producers. However, there is little information about target genes and signaling pathways that are related to LPS exposure in ducklings.

Previous researchers have indicated that the TLR4 pathway plays an important role in pathogen recognition and activation of the innate immune system (Jault et al., 2004). In this pathway, LPS from Gram-negative bacteria is recognized by LPS-binding protein (LBP) and Toll-like receptor (TLR4), which together form a LPS-LBP-TLR4 receptor complex that activates signaling cascades via the nuclear factor-kappa B (NFkB), mitogen-activated protein kinase (MAPK), and interferon regulatory factor (IRF) pathways (Pålsson-McDermott and O'Neill, 2004; Newton and Dixit, 2012). In a study of chickens, researchers found that TLR4 plays an important role in the susceptibility of chickens to systemic salmonellosis (Leveque et al., 2003). Michailidis (2011) also found that chicken TLRs (cTLRs) respond to LPS and *Salmonella enteritidis* (SE).

Zhao (2013) found that the TLR4 gene has two alternative splice forms in duck: TLR4-a and TLR4-b, and both splice forms of TLR4 can be activated by LPS. The authors concluded that the TLR4 signaling pathway is very important for LPS recognition in ducks. However, the role of other genes and pathways that are involved in the immune response of ducklings to LPS remains unknown. Transcription-wide expression analyses could help to determine the key genes

and pathways that play an important role in the immune response of ducklings following treatment with LPS. In this study, a transcriptome-wide expression analysis was performed using RNA-Seq to identify differentially expressed genes and pathways activated in ducklings treated with LPS. A large number of genes and pathways were revealed to be differentially expressed between the LPS-treated and control groups. Our analysis found that the differentially expressed genes and pathways might play an important role in the immune response of ducklings treated with LPS.

MATERIAL AND METHODS

Experimental animals

Ducks, which were provided by Wuhan Tianlv Technological Agriculture Company (P. R. China), were injected with 1000 μg LPS/kg body weight at 7-days of age (N = 6) in the treatment group, and with the same volume of physiological saline in the control group. Twelve-hours later, tissue samples were carefully removed from their spleens, frozen in liquid nitrogen, and stored at -70°C until further use. All research involving animals was conducted according to the relevant regulations (No. 5 proclaim of the Standing Committee of Hubei People's Congress) and approved by the Standing Committee of Hubei People's Congress, and the ethics committee of the Institute of Animal Science and Veterinary Medicine of Wuhan, Wuhan Academy of Agricultural Science & Technology, P.R. China.

RNA isolation and reverse transcription

Spleen samples were placed in 2-mL tubes containing 1 mL TRIzol reagent (Invitrogen, San Diego, CA). One ceramic bead was added immediately to each tube. The tubes were then ground for 30 s using EASY GRIND equipment. Total RNA was extracted using TRIzol according to the manufacturer instructions (Invitrogen, Carlsbad, CA, USA). RNA quality was examined using a ND-1000 Nano-Drop spectrophotometer (Thermo, USA) and by 1.5% agarose gel electrophoresis; gels were stained with 1 μg/mL ethidium bromide.

One microgram total RNA was treated with DNase I (Fermentas, Japan) to remove the contaminating genomic DNA and then reverse transcribed to cDNA using a reverse transcription kit (Fermentas, Japan) according to the manufacturer protocol. The cDNA stocks were stored at -70°C until analysis. A fragment of β-actin that spans an intron sequence was used to verify the efficiency of the reverse transcription reaction and to exclude genomic DNA contamination. The primer sequences were as follows: forward primer, 5'-AACTGGGATGACATGGAGAAGA; and reverse primer, 5'-ATGGCTGGGGTGTTGAAGGT (Li et al., 2012).

RNA-Seq, data mining, and gene ontology (GO) analysis

Spleen RNA from six ducklings treated with LPS were pooled, as were the control samples. Then, Solexa sequencing of the pooled RNA from the LPS-treated samples and the control samples was conducted at Shanghai Hanyu Bio-Tech (Li et al., 2012; Jiang et al., 2013). The Anase Duck database (ftp://ftp.plantbiology.msu.edu/pub/data/Eukaryotic_Projects/o_sativa/annotation_dbs/pseudomolecules/version_6.1/all.dir/) was employed for sequence analysis. A rigorous algorithm was developed by Shanghai Hanyu Bio-Tech based on the methods of Mortazavi and

Wang (2008), and this algorithm was used to identify those genes that were differentially expressed between the two groups. A false discovery rate (FDR) of ≤0.001 was used to identify differentially expressed genes. The differentially expressed genes were analyzed using an MA-plot-based method with the random sampling model of DEGseq. Pathway and GO term analyses were performed using hypergeometric distribution statistics (Wang et al., 2010).

Real-time PCR analysis

Quantitative real-time PCR was performed on a LightCycler 480 real-time PCR thermal cycler instrument (Roche) using SYBR Green qPCR mix (Tubo, Toyota). The real-time PCR system consisted of 10 µL 2X SYBR Green qPCR mix, 0.5 µL cDNA, 8.9 µL double distilled water, and 0.6 mL primer pairs (10 µM forward and 10 µM reverse) in a total volume of 20 µL. Negative controls were performed by replacing the cDNA with double distilled water. The PCR parameters were as follows: denaturation at 95°C for 2 min, followed by 40 cycles of denaturation at 95°C for 20 s, annealing at the appropriate Tm for 20 s, and extension at 72°C for 20 s. Fluorescence was measured using the melting curve program with a heating rate of 2.5°C/s from 57°C to 95°C. All samples were measured three times. The sequences of the quantitative real-time PCR primers were as follows: *CCL4* forward primer, 5'-ACTCTGACCCACCAACCT and reverse primer, 5'-GATTTCACGTCCCTTCCT (Tm = 54°C); *STEAP3* forward primer, 5'-GTCAGTAACAACACCGAAATCA and reverse primer, 5'-TTACCATCCCTGGCACCT (Tm = 54°C); *CD71* forward primer, 5'-TACTTAGCGGATGGTGATG and reverse primer, 5'-TTCTTGTTTGCCCTTTGC (Tm = 60°C); *LBP* forward primer, 5'-TTGCCACTGCCAAGTTCCT and reverse primer, 5'-AGCTGGCGGTGTTGAAGAA (Tm = 55°C); β-actin was used as the reference gene to normalize the quantification of each target mRNA. Primer sequences for β-actin were as follows: forward primer, 5'-AACTGGGATGACATGGAGAAGA and reverse primer, 5'-ATGGCTGGGGTGTTGAAGGT (Tm = 60°C) (Li et al., 2012). The level of relative mRNA expression of the target genes was calculated as fold changes of the threshold cycle (Ct) value relative to the reference gene using the $2^{-\Delta\Delta Ct}$ method. The Ct values of all samples were determined from the mean Ct calculated from the triplicate reactions; thus, ΔCt = Ct (target gene) - Ct (reference gene). Then, the samples were normalized to the calibrator: ΔΔCt = ΔCt (target gene) - ΔCt (calibrator). The calibrator was an untreated control or a particular stage of development to allow for the comparisons of the results (Livak and Schmittgen, 2001). In the present study, one of the control samples was chosen as the calibrator sample to evaluate the putative differential mRNA expression of the target genes. All the data were analyzed using SPSS software 13.0 for Windows (SPSS Inc. Chicago, IL). Data are presented as means ± SE. Differences were considered significant if $P < 0.05$.

RESULTS AND DISCUSSION

Overview of RNA-seq data

To maximize the coverage of duck spleen mRNA by RNA sequencing, libraries were constructed by pooling RNA isolated from the spleens of six LPS-treated ducks to generate the treated library, and six pooled control samples to construct the control library. The RNA-Seq results yielded 7,106,149 (control library) and 7,627,610 (treated library) raw reads. Low quality reads [i.e., tags containing only adaptors and ambiguously called bases (reads that have many Ns)] were

removed. In the control and treated groups, 6,922,339 and 7,401,114 clean reads, respectively, were obtained. The percentage of clean reads was >97%. A total of 5,396,114 and 6,518,449 reads were mapped in the control and treatment groups, respectively. Read analysis showed that 42.65% of the tags in the control library and 39.12% of the tags in the treated library were present in more than 100 reads. The number of genes with more than 10 clean reads was 10,840 and 11,095, respectively. A summary of the sequencing reads and matched genes is shown in Table 1. Information regarding the genes expressed in the control and treated libraries is shown in **Table S1**.

Table 1. A summary of the sequencing reads and matched genes.

Map to genome	Control group		LPS treatment group	
	Read number	Percentage (%)	Read number	Percentage (%)
Clean reads	6,922,339	97.41	7,401,114	97.03
Total mapped reads	5,396,114	75.94	6,518,449	85.46
Total unmapped reads	1,526,225	21.48	882,665	11.57
Unique match	4,145,829	58.34	4,847,888	63.56
Multi-position match	1,250,285	17.59	1,670,561	21.90
Perfect match	2,120,821	29.84	2,541,066	33.31
≤5 bp mismatch	611,489	8.61	787,768	10.33
Genes with more than 100 reads	5,888	42.65	5,363	39.12
Genes with more than 10 clean reads	10,840	78.51	11,095	80.93

Reads: the original sequences sequenced by high-throughput sequencing technology (Sequenced Reads); Clean Reads: the reads that do not have joints or low quality reads; Total mapped reads: the total reads that can mapped to genes in the database; Total unmapped reads: the total reads that cannot be mapped to genes in the database; Unique match: reads can only mapped to one gene in the reference genome database; Multi-position match: reads can be mapped to multi-positions in the reference genome database; Perfect match: there is no mismatch when the reads are mapped to the genes in the reference genome database; ≤5 bp mismatch: ≤5 bp mismatch when the reads are mapped to the genes in the reference genome database.

Differentially expressed genes in LPS-treated, 7-day-old ducks

In this study, a rigorous algorithm was developed by Shanghai Hanyu Bio-Tech, based on the methods of Mortazavi et al. (2008) and Wang et al. (2010), to identify those genes that were differentially expressed between the two samples. These clean reads were mapped to 13,806 and 13,708 genes in the treatment and control groups, respectively. Among these genes, 89 were differentially expressed ($\log_2$ ratio ≥ 1, P ≤ 0.01, FDR ≤ 0.001). Compared to the treated library, the control library showed 67 upregulated and 22 downregulated genes according to the statistical criteria for raw reads and number of transcripts per million clean tags (TMP) (**Table S2**).

Gene ontology analysis, gene ontology richness, pathway analysis, and pathway richness of differentially expressed genes

The genes expressed in the spleens of 7-day-old ducks could be classified into 527 functions using Gene Ontology (GO) analysis (**Table S3**), and GO-rich analysis classified these genes into 58 functions (**Table S4**). Among the functions, the differentially expressed genes were enriched in 13 function categories (P < 0.05), including oxidoreductase activity, metabolism, cell differentiation, integrase activity, extracellular structure organization and biogenesis, extracellular space, enzyme regulator activity, extracellular matrix, antioxidant activity, cell death, pathogenesis, response to stimulus, and extracellular proteins (**Table S4**).

Pathway analysis showed that the differentially expressed genes in the treated and control libraries could be grouped into 327 pathways (**Table S5**). Pathway richness analysis grouped the differentially expressed genes into 39 pathways. The differentially expressed genes in the same pathway are shown in Figure 1. Among the pathways, differentially expressed genes were enriched in six pathway categories ($P < 0.05$), which were as follows: signaling molecules and interaction, transport and catabolism, biosynthesis of other secondary metabolites, metabolism of cofactors and vitamins, immune system, and amino acid metabolism (**Table S6**).

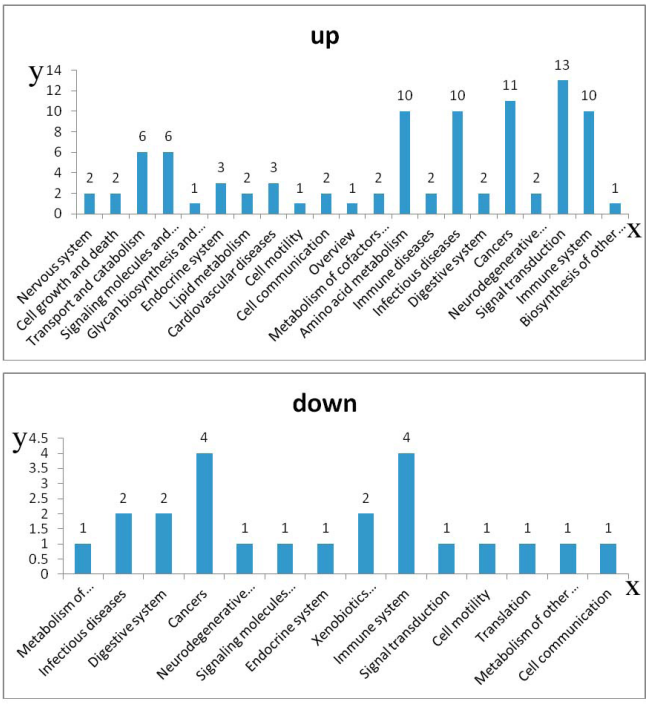

Figure 1. Number of differentially expressed genes in the same pathway (x-axis: pathway, y-axis: the number of differentially expressed genes).

Previous research has shown that TLR signaling pathways play an important role in pathogen recognition and activation of the innate immune system in mammals and chickens (Jault et al., 2004; Zhao et al., 2013). In this way, LPS from Gram-negative bacteria is detected by LBP and TLR4, which form an LPS-LBP-TLR4 complex and subsequently activates signaling cascades via the NF□B, MAPK, and IRF pathways (Pålsson-McDermott and O'Neill, 2004; Newton and Dixit, 2012). Further analysis revealed that some immune system-related signaling pathways are active during the response of ducks to LPS. Apart from TLRs, signaling pathways associated with the immune system include the hematopoietic cell lineage (*MME* [membrane metallo-endopeptidase] and *CD71*[TFRC, transferrin receptor]), T cell receptor signaling pathway (*ITK* [IL2-inducible T-cell kinase]), complement and coagulation cascades (*C1R* [complement component 1, r subcomponent] and *C7* [complement component 7]), antigen processing and presentation (*CTSB* [cathepsin B]), and chemokine signaling pathway (*CCL4*, *CCLX* [C-C motif chemokine], other), *IL8RB* (interleukin 8 receptor beta), and *ITK* (IL2-inducible T-cell kinase).

The hematopoietic cell lineage pathway is involved in the developmental progress of the lymphoid lineage of white blood cells or leukocytes, the natural killer (NK) cells and the T and B lymphocytes from hematopoietic stem cells (HSC) (Di Santo et al., 1999; Rothenberg and Taghon, 2005). The T cell receptor signaling pathway is mainly associated with T-cell proliferation, cytokine production, and differentiation into effector cells. The activation of T lymphocytes is essential for an efficient immune response (Huang and Wange, 2004; Smith-Garvin et al., 2009). The activation of hematopoietic cell lineage and T cell receptor signaling pathways may indicate that T lymphocytes are proliferating and that cytokines are being produced in response to Gram-negative bacterial infection in ducklings.

The complement and coagulation cascades are key pathways in the complement system, which is a proteolytic cascade and a mediator of innate immunity, and a nonspecific defense mechanism against pathogens. The activation of this pathway leads to the opsonization of pathogens, recruitment of inflammatory and immunocompetent cells, and the direct killing of pathogens (Ruf et al., 2003; Turnberg and Botto, 2003). Based on our results, we hypothesize that the complement system participates in the immune response to Gram-negative bacterial infection in ducklings.

An inflammatory immune response is dependent on the recruitment of leukocytes to the site of inflammation following the detection of a foreign insult. During an inflammatory immune response, the chemokine signaling pathway is activated and transduced by chemokine receptors (G-protein coupled receptors) expressed on leukocytes. After chemokine receptor activation, the α- and βγ-subunits of the G protein dissociate to activate diverse downstream pathways that result in cellular polarization and actin reorganization (Wong and Fish, 2003; Thelen and Stein, 2008). The activation of chemokine signaling pathways suggests that chemokines are produced to provide directional cues for lineage determination in lymphocyte cells following the infection of ducklings with Gram-negative bacteria.

An overview of genes related to the immune system is shown in Table 2. LBP, IL8RB, CD71, C1R, C7, CTSB, CCLX, IL8RB, and CCL4 were upregulated in the treated library compared with the control library, while MME and ITK were downregulated (Table 2). As we know, ducklings have a weaker defense and immune response to Gram-negative bacterial diseases than adult ducks due to their immature immune systems. Our results indicate that the six signaling pathways could cooperatively participate in response to Gram-negative bacterial infection in ducklings. This information is useful for researchers and duck producers who aim to prevent Gram-negative bacteria prevention in duckling.

Table 2. A summary of the genes related to the immune system that were identified by RNA-Seq.

Genes	Treatment		Control		Treatment/Control		
	Read	RPM	Read	RPM	log2 (Treatment/Control)	q-value	Result
CD71	3287	981.329	1070	389.446	1.333	3.55E-174	Up
CCL4	630	188.086	106	38.581	2.285	4.04E-70	Up
C1R	318	94.938	89	32.393	1.551	6.80E-21	Up
C7	498	148.677	120	43.676	1.767	1.52E-39	Up
LBP	3593	1072.685	241	87.716	3.612	0	Up
CTSB	5604	1673.066	1933	703.551	1.250	6.33E-269	Up
CCLX	309	92.252	122	44.404	1.055	3.28E-11	Up
IL8RB	84	25.078	31	11.283	1.152	0.0006956	Up
MME	508	151.663	994	361.785	-1.254	4.86E-58	Down
ITK	84	25.078	139	50.592	-1.012	5.56E-06	Down

RPM = reads per kilobases per million reads. q-value = FDR (False discovery rate) is used to determine the threshold P value in multiple tests and analyses by manipulating the FDR value. q-value < 0.001 means the differentially expressed genes were significantly different (Benjamini et al., 2001).

Real-time PCR confirmation of differential gene expression in ducks

To confirm the differential gene expression observed by RNA-Seq, we measured the expression of *LBP*, *CCL4*, and *CD71*, which are involved in immune function, and the *STEAP3* gene, which is involved in cell growth and death, in the spleens of LPS-treated ducks using quantitative real-time PCR analysis. Those genes were selected from differentially expressed genes as determined from the RNA-Seq data. The samples were treated in the same way as those used for RNA-Seq.

Results of real-time PCR analysis revealed that *LBP* is highly expressed after LPS treatment in ducklings (Figure 2A). LBP is a crucial molecule involved in the innate immune response to a bacterial challenge (Jault et al., 2004; Barton and Kagan, 2009; Kawai and Akira, 2010; Ding et al., 2013, Hsu et al., 2013). These results indicate that the TLR signaling pathway participates in the immune response to Gram-negative bacterial infection in ducklings.

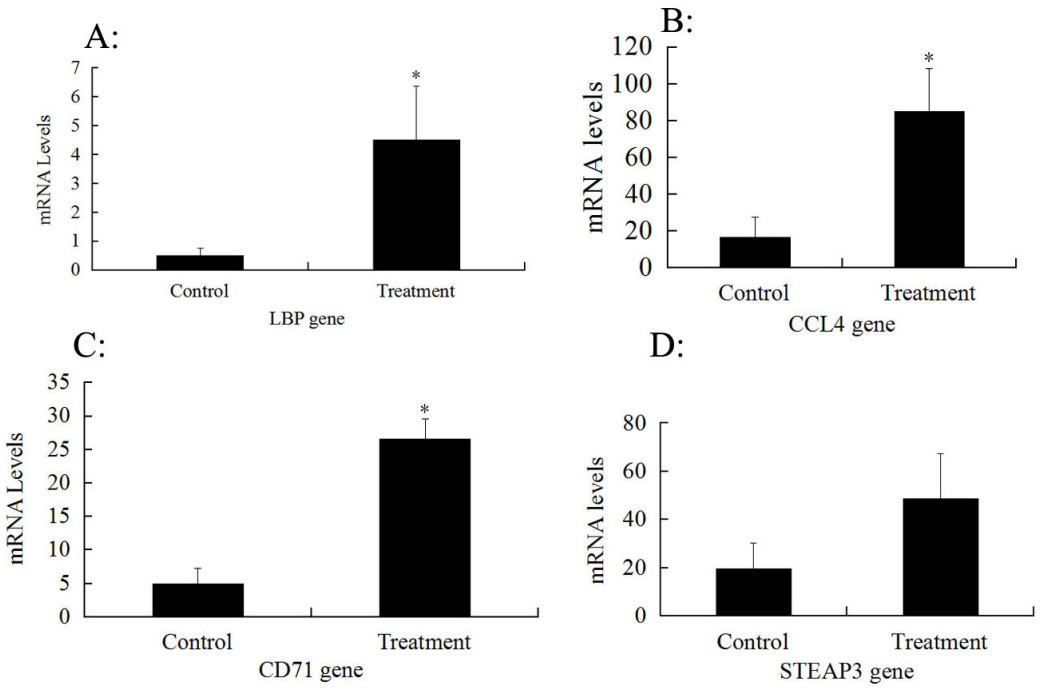

Figure 2. Confirmation of differential gene expression in control and LPS-treated animals. **A.** *LBP* mRNA expression was measured by real-time PCR analysis in control and LPS-treated animals. **B.** *CCL4* mRNA expression was measured by real-time PCR analysis in control and LPS-treated animals. **C.** *CD71* mRNA expression was measured by real-time PCR analysis in control and LPS-treated animals. **D.** *STEAP3* mRNA expression was measured by real-time PCR analysis in control and LPS-treated animals. Asterisk denotes a significant difference ($P < 0.05$).

CC chemokine (motif) ligand 4 (CCL4) is indispensable for the chemoattraction of macrophages, natural killer cells, and lymphocytes in mammals. In LPS-stimulated fish, CCL4 expression did not increase until nearly 24 h after induction (Hsu et al., 2013). However, our results indicated that *CCL4* was significantly upregulated in ducklings following treatment with LPS for 12 h (Figure 2B). This shows that this gene is involved in the inflammatory response in the spleens of ducklings.

The CD71 (transferrin receptor) protein, which is closely associated with activation, proliferation, and differentiation, is expressed in liver cells, red blood cells, and lymphocytes along with many other cell surface proteins (Gutsol et al., 2013). The results show that *CD71* gene expression was upregulated after treatment with LPS, which suggests that the function of *CD71* is associated with the proliferation of spleen lymphocytes (Figure 2C).

The *STEAP3* gene is part of the p53 signaling pathway and has a role in cell growth and death signaling, and is involved in the TLR4-mediated inflammatory response in macrophages (Zhang et al., 2012). Results from real-time PCR and RNA-Seq analysis showed that *STEAP3* gene expression is upregulated after treatment with LPS, indicating that the product of this gene may be involved in the inflammatory response in duckling spleens (Figure 2D).

In this study, we performed transcription-wide expression analysis using RNA-Seq to identify genes that are differentially expressed in response to LPS treatment in ducks. Results indicated that 89 genes were differentially expressed; 67 of these genes were upregulated and 22 were downregulated during the immune response of ducklings to LPS. Our results also provide solid evidence for some of the functional mediators involved in mediating the response to LPS in ducklings, such as the hematopoietic cell lineage, TLR signaling pathways, T cell receptor signaling pathways, complement and coagulation cascades, antigen processing and presentation, and chemokine signaling pathways.

Conflicts of interest

The authors declare no conflict of interest.

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Supplementary material

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