Fish and Shellfish Immunology 92 (2019) 842–850

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Differential expression of immune-related genes in head kidney and spleen of cobia (*Rachycentron canadum*) having *Streptococcus dysgalactiae* infection

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ARTICLE INFO

Keywords:
RNA-seq
Immune response
Cobia (Rachycentron canadum)
Streptococcus dysgalactiae
Head kidney
Spleen

ABSTRACT

Streptococcus dysgalactiae is a gram-positive bacterium and a harmful aquaculture pathogen. To investigate the immune response against *S. dysgalactiae*, we performed transcriptome analysis of the head kidney and spleen of cobia (*Rachycentron canadum*) using RNA-seq. Total RNA was extracted from the head kidney and spleen of cobia, 1 and 2 days after treatment with *S. dysgalactiae* or control PBS. After RNA purification and cDNA library generation, sequencing was performed using the Illumina HiSeq™ 4000 platform. The filtering and *de novo* assembling transcripts were annotated using several databases. To identify differentially expressed genes (DEGs) between the *S. dysgalactiae* and PBS groups, the mapped values of fragments per kilobase of transcripts per million fragments were calculated. After *de novo* assembly, a total of 106,984 transcripts were detected, with an N50 of 3020 bp. These transcripts were annotated and categorised into a total of 7608 genes based on the KEGG pathway database. DEGs (2-fold difference) were calculated by comparing the *S. dysgalactiae* and PBS control group gene expression levels at each time point. The DEGs were mainly annotated into signal transduction and immune system categories, based on the KEGG database. The DEGs were significantly enriched in the immune-related pathways – "cytokine-cytokine receptor interaction", "complement and coagulation cascades", and "hematopoietic cell linage". In this study, immune-related genes responding to *S. dysgalactiae* were detected, and several immune system pathways were categorized. We identified the IL17C-related pathway for inducing the expression of pro-inflammatory cytokine genes (IL-1β, IL-6, and IFNγ). Additionally, neutrophil-related genes (CSF3, CD121, and CD114) were induced in the spleen after *S. dysgalactiae* infection. It was suggested that these pathways contribute to immune responses against *S. dysgalactiae* infection. The data revealed in this study may offer improved strategies against *S. dysgalactiae* infection in cobia.

1. Introduction

Transcriptome has been used to investigate the key pathways of development, cellular fate, physiology, activity, and disease progression. RNA sequencing (RNA-seq) by next-generation sequencing (NGS) has become a modern technology for transcriptome analysis. Moreover, developments of bioinformatics has strongly supported RNA-Seq technology [1]. The advantage of RNA-seq in the aquaculture field is to examine any organisms that do not have reference gene sequences [2]. In the aquaculture field, there is many reports using RNA-Seq in various species and applications including immunology studies. RNA-seq has been performed to understand the immune mechanisms in various aquaculture fish during various pathogenic infections. These transcriptome profiling have shown the diversity of immune-related genes in different fish species after bacterial infection [3]. Therefore, it is important to increase the transcriptome knowledge of immune response in various fish species against various pathogens. *Streptococcus dysgalactiae* is a Gram-positive bacteria, and is a causative agent of mastitis n

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https://doi.org/10.1016/j.fsi.2019.07.009
Received 31 May 2019; Received in revised form 4 July 2019; Accepted 5 July 2019
Available online 06 July 2019
1050-4648/

cattle and pharyngitis in humans [4,5]. *S. dysgalactiae* have been identified in various fish species, such as amberjack (*Seriola dumerili*) and yellowtail (*Seriola quinqueradiata*) in Japan [6], and cobia (*Rachycentron canadum*) and gray mullet (*Mugil cephaleus*) in Taiwan [7,8]. Spontaneously affected cobia initially showed clinical signs of anorexia without external lesions. In the gross pathology, conjunctival haemorrhage, hepatic haemorrhage, hyperaemia in brain, ascites, and pericarditis were observed [7]. Additionally, cobia infected with *S. dysgalactiae* showed the necrotic foci and bacterial clumps mainly in spleen and kidney, and modelately in heart [7]. Recently, transcriptome analysis was performed in the spleen of soiny mullet (*Liza haematocheila*) infected with *S. dysgalactiae*, and demonstrated that several immune-related pathways were upregulated in the spleen at 1 day post-infection [9]. It is still unclear the immune responses in cobia against *S. dysgalactiae* and its universality and diversity among aquaculture species. The purpose of this study is identification of immune related genes and pathways in spleen and head kidney in cobia (*Rachycentron canadum*) infected with *Streptococcus dysgalactiae*. In current study, we performed transcriptome profiling analysis of the head kidney and spleen of cobia (*Rachycentron canadum*) infected with *S. dysgalactiae* using NGS. The results of transcriptome sequences and differentially expressed genes in this study offer a valuable resource for further research and effective strategies against *S. dysgalactiae*.

2. Materials and methods

2.1. Animals

Healthy cobia (*Rachycentron canadum*) without pathogenic infection were used in this study (average body weight 85 g). The fish were maintained in an outdoor facility at a water temperature of 28 °C. We acclimatised the fish for two weeks before experiments. All experiments in this study were conducted according to the Centre for Research Animal Care and Use Committee of the National Pingtung University of Science and Technology (NPUST-106-067).

2.2. Streptococcus dysgalactiae infection

Before infection, 25 fish were anaesthetised with 2-phenoxyethanol and intraperitoneally injected with a non-lethal dose of 1.0×10^6 cfu *Streptococcus dysgalactiae* (SD12 strain) [10], suspended in 100 μL phosphate-buffered saline (PBS). For control group, another 25 fish were injected with 100 μL PBS. The head kidney and spleen were sampled from 8 individual fish each from the infection and control groups at 1 and 2 days post-infection (dpi).

2.3. Total RNA extraction, cDNA library preparation, and sequencing

Total RNA was extracted using TRIzol® reagent (Invitrogen Corp., Carlsbad, CA, USA). Two micrograms of total RNA from each of the eight samples were pooled in each experimental group to average the expression levels of individual fish in RNA-seq analysis. The quality of the total RNA was assessed using an Agilent Bioanalyzer 2100 system (Agilent Technologies, Palo Alto, CA, USA). For generating cDNA libraries, the total RNA were purified using TruSeq™ RNA Sample Preparation Kit (Illumina, Inc., San Diego, CA, USA). First-strand cDNA was synthesized by Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) with random hexamer primers. Subsequently, second-strand cDNA synthesis, end repair, and ligation of the adaptors were performed. Sequencing was performed using the Illumina HiSeq™ 4000 platform (Illumina, Inc., San Diego, CA, USA) and 150 bp paired-end reads generated at Genomics Bioscience Technology Co., Ltd. (Taipei, Taiwan).

Table 1
Length distribution and quality matrix of assembled transcripts.

Sample	Total Number	Total Length	Mean Length	N50	N70	N90	GC(%)
PBS_1dpi_kidney	71,956	95,111,956	1321	2455	1567	533	45.82
PBS_1dpi_spleen	67,165	80,864,004	1203	2166	1349	480	46.98
PBS_2dpi_kidney	56,047	65,021,460	1160	2072	1280	459	47.33
PBS_2dpi_spleen	70,109	86,644,519	1235	2208	1377	499	47.10
SD_1dpi_kidney	67,996	84,932,496	1249	2263	1427	503	46.60
SD_1dpi_spleen	65,876	77,589,620	1177	2076	1293	473	47.20
SD_2dpi_kidney	63,067	77,502,173	1228	2214	1393	494	47.12
SD_2dpi_spleen	69,462	83,368,994	1200	2134	1338	482	47.00
All-Unigene	106,984	183,708,480	1717	3020	2047	857	46.77

SD, *S. dysgalactiae* injection. N50, N70, and N90: a weighted median statistic that 50, 70 and 90%, respectively, of the total length is contained in transcripts great than or equal to this value. GC (%): the percentage of G and C bases in all transcripts.

Table 2
The number of functional annotation transcripts.

Values	Number	Percentage
Total Unigenes	106,984	100%
NR-Annotated	68,640	64.16%
NT-Annotated	74,521	69.66%
Swissprot-Annotated	62,299	58.23%
KEGG-Annotated	56,041	52.38%
COG-Annotated	20,324	19.00%
GO-Annotated	31,133	29.10%
Overall	78,651	73.52%

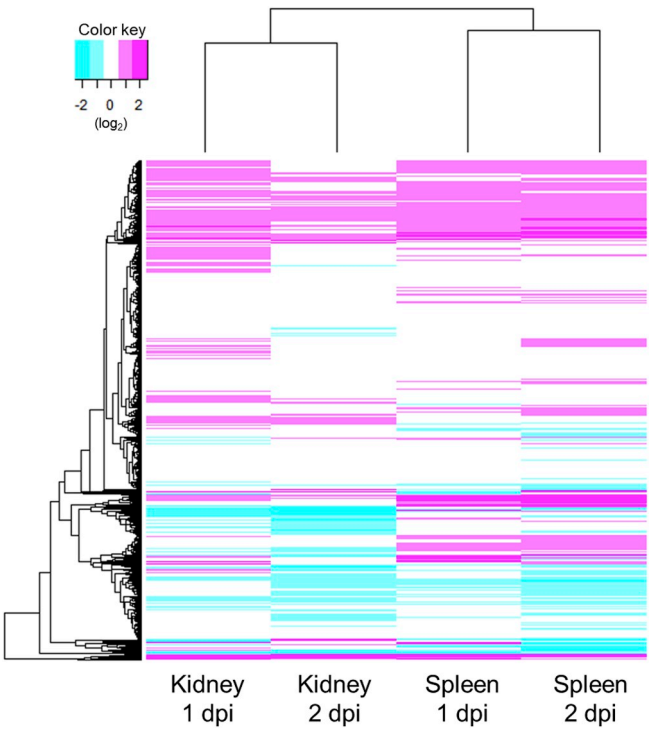

Fig. 1. Clustering by gene expression profiles in a transcriptomic dataset. Scores are colored on a $\log_2$ scale (*S. dysgalactiae* infection/PBS treatment) with magenta maximum and cyan minimum. Heat maps and clustering were generated using the gplots package of R statistical program. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

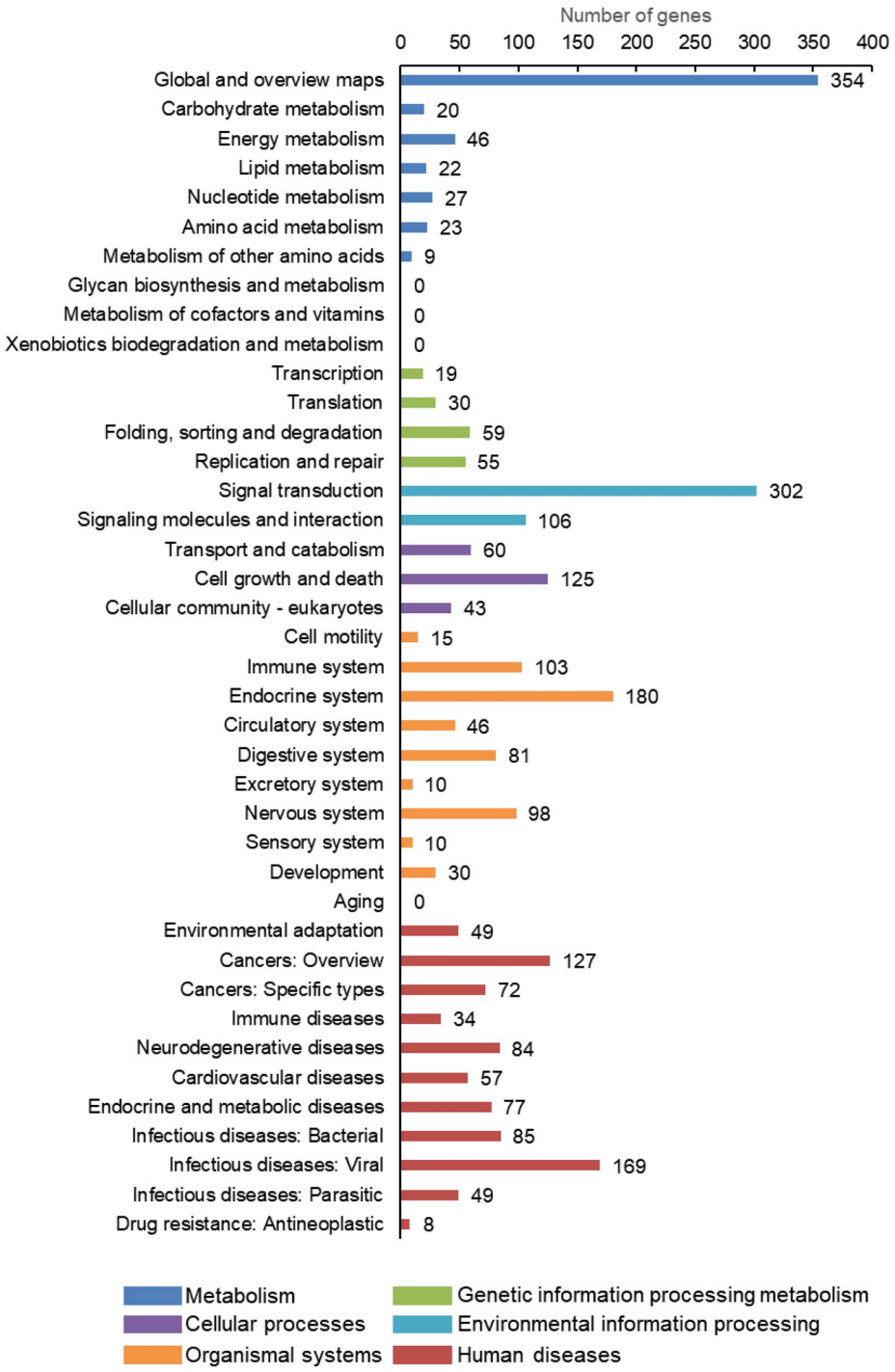

Fig. 2. Distribution of differentially expressed genes from 2 dpi of spleen in KEGG pathway analysis. KEGG enrichment analysis of differently expressed genes were classified into six categories: metabolism, genetic information processing, cellular processes, environmental information processing, organismal systems, and human diseases. The X-axis indicated the name of functional classification in KEGG pathway, and the Y-axis identifies the numbers of annotated genes.

2.4. Reads data filtration and de novo transcriptome assembly

To obtain clean data of sequences, low quality reads (containing adapter and poly-N) were removed from raw data, and the Phred quality score (Q20 and Q30) [11], and GC-content of the clean data were calculated. These clean data were stocked in FASTQ file format [12]. After filtering read data, *de novo* assembly was performed using Trinity [2] and finally TIGR Gene Indices clustering tools (Tgicl; version v2.0.6) to establish a final set of Unigenes [13].

2.5. Annotation of transcripts sequences

The assembled transcripts were functionally annotated with the following databases: NCBI nucleotide sequences (NT), NCBI non-redundant protein (NR), Clusters of Orthologous Groups (COGs), the Kyoto Encyclopedia of Genes and Genomes (KEGG) using BlastP (Version 2.2.25) [14], and gene ontology (GO) using Blast2GO (version v2.5.0) [15].

Table 3
Functional enrichment KEGG pathway for DEGs in 2 dpi kidney.

Pathway ID	Pathway Name	Count	(%)	Q-value
ko04080	Neuroactive ligand-receptor interaction	49	(5.73)	7.3E-09
ko04610	Complement and coagulation cascades	18	(2.11)	4.3E-04
ko04979	Cholesterol metabolism	15	(1.75)	3.8E-04
ko00260	Glycine, serine and threonine metabolism	14	(1.64)	4.5E-04
ko04924	Renin secretion	16	(1.87)	6.7E-04
ko05150	*Staphylococcus aureus* infection	11	(1.29)	1.1E-03
ko04911	Insulin secretion	17	(1.99)	1.5E-03
ko04060	Cytokine-cytokine receptor interaction	33	(3.86)	1.9E-03
ko00860	Porphyrin and chlorophyll metabolism	10	(1.17)	1.8E-03
ko00950	Isoquinoline alkaloid biosynthesis	5	(0.58)	3.9E-03
ko05144	Malaria	12	(1.40)	4.7E-03
ko04934	Cushing syndrome	25	(2.92)	5.7E-03
ko00350	Tyrosine metabolism	8	(0.94)	8.2E-03
ko04976	Bile secretion	14	(1.64)	8.0E-03
ko04640	Hematopoietic cell lineage	15	(1.75)	9.5E-03
ko04927	Cortisol synthesis and secretion	14	(1.64)	1.2E-02
ko04261	Adrenergic signaling in cardiomyocytes	19	(2.22)	1.4E-02
ko05410	Hypertrophic cardiomyopathy (HCM)	14	(1.64)	1.7E-02
ko05414	Dilated cardiomyopathy (DCM)	15	(1.75)	2.2E-02
ko00360	Phenylalanine metabolism	5	(0.58)	3.0E-02
ko04022	GMP-PKG signaling pathway	22	(2.57)	3.5E-02
ko04940	Type I diabetes mellitus	7	(0.82)	4.4E-02
ko04978	Mineral absorption	8	(0.94)	4.9E-02

Table 4
Functional enrichment KEGG pathway for DEGs in 2 dpi spleen.

Pathway ID	Pathway Name	Count	(%)	Q-value
ko03050	Proteasome	29	(2.08)	4.7E-10
ko04974	Protein digestion and absorption	24	(1.72)	4.9E-06
ko03030	DNA replication	18	(1.29)	3.7E-04
ko04966	Collecting duct acid secretion	10	(0.72)	4.7E-04
ko00190	Oxidative phosphorylation	38	(2.72)	1.1E-03
ko04111	Cell cycle - yeast	24	(1.72)	1.7E-03
ko05150	*Staphylococcus aureus* infection	13	(0.93)	1.9E-03
ko05323	Rheumatoid arthritis	20	(1.43)	4.4E-03
ko04060	Cytokine-cytokine receptor interaction	44	(3.15)	5.0E-03
ko01200	Carbon metabolism	29	(2.08)	5.8E-03
ko04110	Cell cycle	31	(2.22)	1.7E-02
ko00680	Methane metabolism	8	(0.57)	2.6E-02
ko00590	Arachidonic acid metabolism	11	(0.79)	3.6E-02
ko04975	Fat digestion and absorption	9	(0.64)	3.9E-02
ko05321	Inflammatory bowel disease (IBD)	14	(1.00)	4.1E-02
ko04260	Cardiac muscle contraction	18	(1.29)	4.3E-02
ko00260	Glycine, serine and threonine metabolism	13	(0.93)	4.3E-02
ko05144	Malaria	13	(0.93)	4.0E-02
ko05412	Arrhythmogenic right ventricular cardiomyopathy (ARVC)	16	(1.15)	4.1E-02
ko04080	Neuroactive ligand-receptor interaction	43	(3.08)	4.7E-02
ko01120	Microbial metabolism in diverse environments	34	(2.43)	4.7E-02
ko01230	Biosynthesis of amino acids	16	(1.15)	4.5E-02
ko04721	Synaptic vesicle cycle	16	(1.15)	4.3E-02
ko04976	Bile secretion	16	(1.15)	4.2E-02

2.6. Differentially-expressed genes and functional enrichment analysis

The expression data of each transcript were obtained using Bowtie2 software (version v2.2.6) [16], and the values of fragments per kilobase of transcripts per million fragments mapped (FPKM) were calculated using RSEM (version v1.2.12) [17]. We detected the differentially expressed genes (DEGs) with edgeR software [18,19]. Differentially expressed genes were determined by 2-fold difference in expression levels between the *Streptococcus dysgalactiae* infection group and the PBS group. For KEGG pathway enrichment analysis, we classified DEGs according to KEGG classification and calculated the *p*-value using a hypergeometric test. We then calculated the false discovery rate (FDR) for each *p*-value, and an FDR less than 0.05 was defined as significant enrichment.

2.7. Real-time reverse transcription polymerase chain reaction

One microgram of total RNA treated by DNase I was subjected to cDNA synthesis using iScript™ cDNA synthesis kits (Bio-Rad). Reverse transcriptase real-time PCR (RT-qPCR) was performed using iQ™ SYBR® Green Supermix (Bio-Rad). To calculate relative expression levels, the mean threshold cycle was used, and the gene expression levels were normalised by mall subunit ribosomal protein S6e (RPS6) gene expression. We selected RPS6 as internal control based on previously reports [20,21] and RNA-seq data in this study, which showed that the relative expression levels were less than 2-fold differences in each groups. Primers were designed using Primer3 software [22] and listed in Table S1. Statistical analysis was performed using a Student' *t*-test to compare between the two groups. A *p*-value of less than 0.05 was considered statistically significant.

3. Results

3.1. Assembly and functional annotation of read transcript sequences

In this study, transcriptome analysis using an Illumina HiSeq™ 4000 platform was performed on the head kidney and spleen samples from two groups with *Streptococcus dysgalactiae* or PBS injection as a control. After removing low quality sequences, over 55 M clean reads were generated with a high percentage of Q20 and Q30 values in all samples (Table S2). Using *de novo* assembly, a total of 106,984 unigenes were obtained with an N50 length of 3020 bp and GC content of 46.77% (Table 1). The unigenes were predicted using identified sequences of genes in the databases, NT, NR, Swiss-Prot, COG, KEGG, and GO. A total of 78,651 (73.52%) of the unigenes were annotated using at least one database, containing 68,640 (64.16%) in NR, 74,521 (69.66%) in NR, 62.299 (58.23%) in Swiss-Prot, 56,041 (52.38%) in KEGG, 20,324 (19.00%) in COG, and 31,133 (29.10%) in GO (Table 2).

3.2. Differentially expressed genes after Streptococcus dysgalactiae challenge

Prior to calculating expression levels, we clustered the unigenes based on KEGG annotation [23]. As a result, a total of 7230 genes were obtained. Differentially expressed genes (DEGs) in each sample and days post-infection (dpi) were shown by the relative expression level of genes in *Streptococcus dysgalactiae* when compared to the PBS-treated groups. The number of total DEGs in the head kidney at 1 dpi, head kidney at 2 dpi, spleen at 1 dpi, and spleen at 2 dpi were 832 (526 upregulated and 306 downregulated genes), 855 (298 upregulated and 557 downregulated genes), 896 (716 upregulated and 180 downregulated genes), and 1397 (888 upregulated and 509 downregulated genes), respectively. Clustering analysis using a heat map revealed categorisation by tissue, rather than days post-infection (Fig. 1). Next, we performed classification and functional enrichment for DEGs based on the KEGG pathway. DEGs were annotated into 6 main categories, including metabolism, genetic information processing, metabolism, cellular processes, environmental information processing and organismal system, and human diseases. (Fig. 2, Fig. S1). Generally, for each sample, DEGs were mainly categorised as "global and overview maps" under metabolism, "folding, sorting and degradation" under genetic information processing metabolism, "signal transduction" under environmental information processing, "cell growth and death" under cellular processes, "immune system" under organismal systems, and "neurodegenerative diseases and infectious diseases: viral" under the human diseases category. In the head kidney sample at 1 dpi (Table S3), DEGs were significantly assigned to 4 KEGG pathways, which included proteasome (30 genes, 3.61%), biosynthesis of amino acids (15 genes, 1.80%), arginine biosynthesis (7 genes, 0.84%), and malaria (11 genes, 1.32%). In the head kidney sample at 2 dpi (Table 3), 23 KEGG

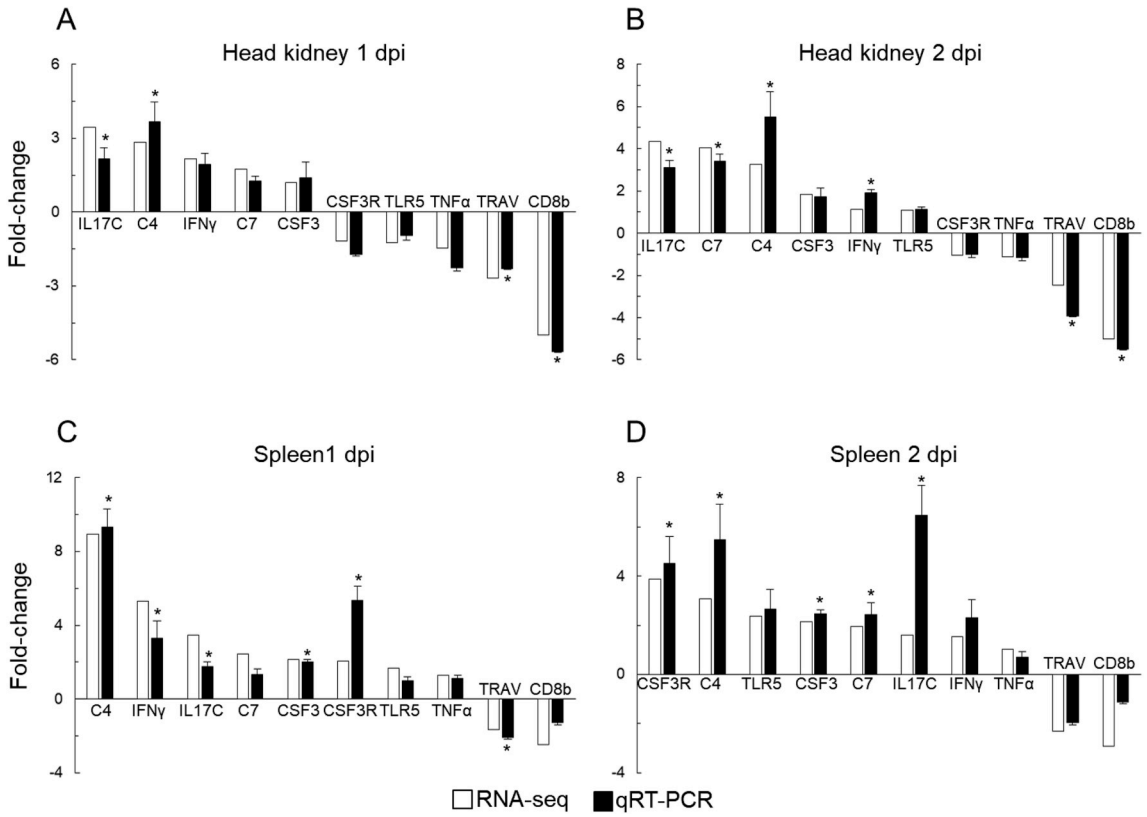

Fig. 3. Gene expression analysis from RNA-Seq and RT-qPCR of cobia infected with *Streptococcus dysgalactiae*. Total RNA from head kidney and spleen were extracted, and expression levels of each genes determined by RT-qPCR. Expression levels of target genes were normalized by RPS6 as a reference gene. The graph shows the relative expression level of the *S. dysgalactiae* infection group and PBS-treated groups. (n = 8, *p < 0.05, compared to PBS-treated group).

pathways were significantly assigned by DEGs. According to the immune system classification, DEGs were classified into 3 pathways, which included complement and coagulation cascades (18 genes; 2.11%), cytokine-cytokine receptor interaction (33 genes; 3.86%), and hematopoietic cell lineage (15 genes; 1.75%). In the spleen sample at 1 dpi (Table S4), 12 KEGG pathways were significantly assigned by DEGs. According to the immune system classification, DEGs were classified into 3 pathways, which included cytokine-cytokine receptor interaction (31 genes; 3.46%), phagosome (20 genes; 2.23%), and *Vibrio cholerae* infection (11 genes; 1.45%). In the spleen sample at 2 dpi (Table 4), 24 KEGG pathways were significantly assigned by DEGs. According to the immune system classification, DEGs were classified into 2 pathways, which included cytokine-cytokine receptor interaction (44 genes; 3.15%) and inflammatory bowel disease (IBD) (14 genes; 1.00%).

3.3. Validation of differentially expressed gene by RT-qPCR

We identified mRNA sequences of immune-related genes that were either up or downregulated by *Streptococcus dysgalactiae* infection, based on RNA-seq data using the NCBI database. The expression levels of DEGs, C4, C7, CD8B, CSF3, CSF3R, IFNγ, IL17C, TNFα, TRAV, and TLR5 in immune-related pathways, including cytokine-cytokine receptor interaction, hematopoietic cell lineage, and complement and coagulation cascades, were determined in the head kidney and spleen using RT-qPCR (Fig. 3). The expression level of each gene was mainly consistent with the RNA-seq data. These results suggested that the transcriptome data were reliable (Fig. 3).

3.4. Analysis of immune-related pathways

KEGG pathways and DEGs were determined to be involved in the signaling pathways related to the immune system. Fig. 4 shows the complement cascade in the KEGG database (ko04610), and suggests that *Streptococcus dysgalactiae* infection induced the expression of C2, C4, and C7 genes. Meanwhile, MBL and MASP1, which are the origin of the lectin pathway, were downregulated after infection. In the cytokine-cytokine receptor interaction (ko04060) pathway, many immune-related genes were identified (Table S5). The results of RNA-seq indicated that *S. dysgalactiae* infection influenced the gene expression of immune-related cytokines (IL-1β, IL-6, IL-12A (p35 subunit), IFNγ, and IL17C) and chemokines (CCL19 and CCL21). Additionally, IL-17 signaling pathway (ko04657) showed that upregulated IL-17C induced IL-1β, IL-6, and IFNγ expression (Fig. 5A). We also investigated the hematopoietic cell lineage (ko04640) pathway, as assigned by DEGs (Fig. 5B and C). Fig. 5B indicated that neutrophil-related genes (CSF3, CD121, and CD114) were upregulated in the spleen after *S. dysgalactiae* infection. However, in Fig. 5C, the expression levels of T-cell-related genes (CD127, CD4, CD8, and CD3) were downregulated in the head kidney and spleen after *S. dysgalactiae* infection.

3.5. ARG2 is a common factor in neutrophil and T-cell proliferation

Many reports have shown that neutrophils act as effectors in both innate and adaptive immune cells, including T-cells [24]. We therefore investigated the key regulator of T-cell downregulation by neutrophil activation. Based on the GO ontology database, we firstly identified the DEGs related to neutrophil degranulation (GO: 0043312) and negative regulation of T-cell proliferation (GO: 0042130 and GO: 0046007). In Fig. 6A and B, we found that ARG2 gene upregulation was commonly categorized under neutrophil degranulation and negative regulation of T-cell proliferation. Finally, we validated the expression level of the ARG2 gene after *Streptococcus dysgalactiae* infection by RT-qPCR. In each group, the expression levels of ARG2 genes were almost consistent with the RNA-seq data (Fig. 6C).

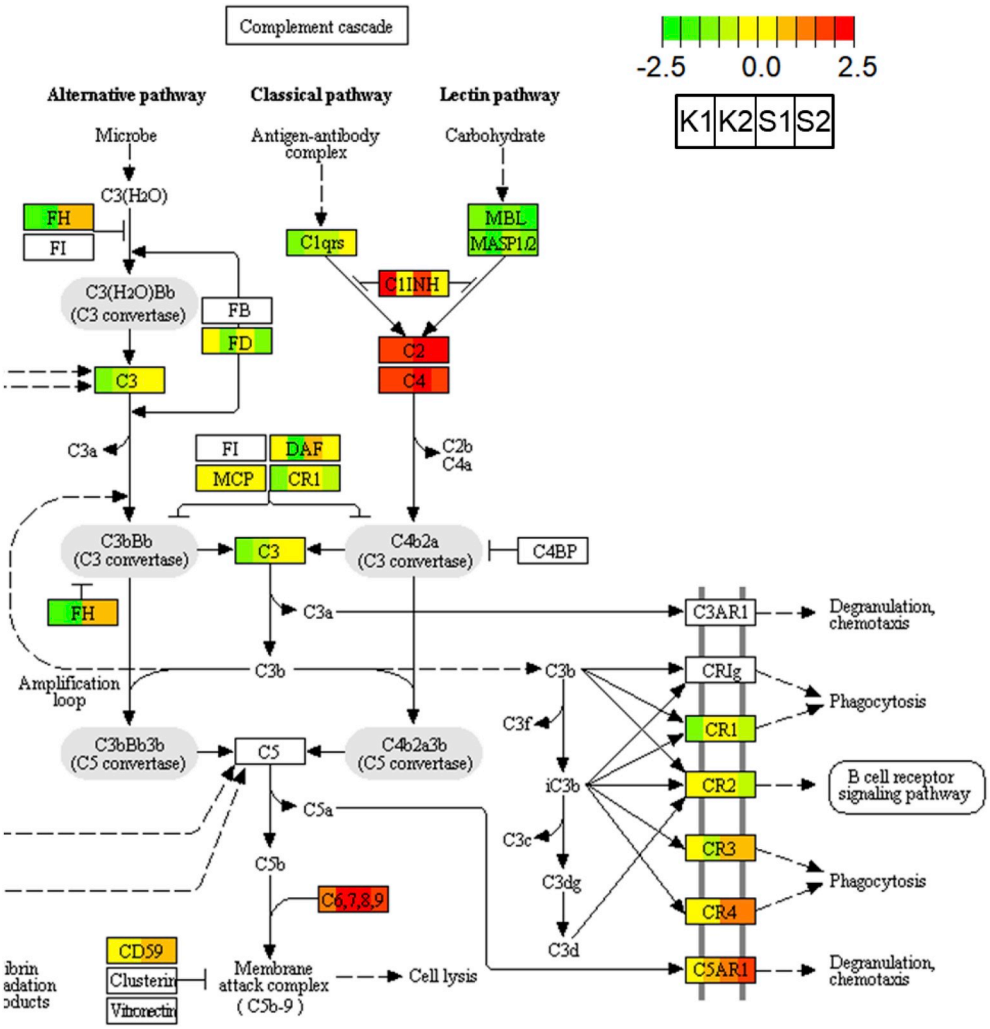

Fig. 4. Pathway map of complement pathway in KEGG. The relative expression levels compared *Streptococcus dysgalactiae* infection group and PBS-treated group, are shown in this illustration of complement pathway in KEGG database (ko04610). In each gene boxes, the gene expression levels are shown in 4 group (K, kidney; S spleen; 1, 1 dpi; 2, 2 dpi). The lower expression levels of genes are shown in green, and the higher expression levels of genes are shown in red. Undetected genes are shown by white coloring (see color legend in figure). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

4. Discussion

In this study, Illumina RNA sequencing was used for transcriptome analysis of the head kidney and spleen of cobia (*Rachycentron canadum*) at 1 and 2 days post-*Streptococcus dysgalactiae* infection. We obtained a total of 106,984 unigenes using *de novo* assembly, and observed immune-related DEGs.After *de novo* assembly, a total of 106,984 unigenes were identified with an N50 length of 3020 bp and 46.77% GC content (Table 1). After homology alignments with the major databases NT, NR, Swiss-Prot, COG, KEGG, and GO, a total of 78,651 (73.52%) of the unigenes were annotated using at least one database (Table 2). In this study, we clustered the unigenes based on KEGG annotation for further analysis, and obtained a total of 7230 genes. The total numbers of DEGs were 832 (526 upregulated and 306 downregulated genes) in the head kidney at 1 dpi, 855 (298 upregulated and 557 downregulated genes) in the head kidney at 2 dpi, 896 (716 upregulated and 180 downregulated genes) in the spleen at 1 dpi, and 1397 (888 upregulated and 509 downregulated genes) in the spleen at 2 dpi. Clustering analysis using a heat map revealed that categorization was done by tissue, rather than by days post-infection (Fig. 1). DEG clustering analysis suggested that the immune response against infection is different between the kidney and spleen. In each sample, DEGs were mainly categorized under immune system-related pathways and signal transduction pathways (Fig. 2, Fig. S1). The complement system is one of the major innate immune systems in vertebrates, including teleosts [25]. The expression levels of C2, C4, and C7 were upregulated at 1 and 2 dpi in the head kidney and spleen, compared with the control group (Fig. 4). The complement system pathway in KEGG showed that these upregulated genes were linked to the membrane attack complex, which damages the bacterial membrane [26]. Previously, we found that the complement system commonly contributes to early immune responses following bacterial infection, based on several transcriptome data [3]. Therefore, the complement system is also a primary immune response system in cobia infected with *Streptococcus dysgalactiae*. The expression of typical inflammatory cytokines (IL-1β, IL-6, IL-12A and IFNγ) were upregulated in the head kidney and spleen after *Streptococcus dysgalactiae* infection (Table S5). These results demonstrated that early immune systems were activated by the infection in cobia. We also found enhancement of IL-17C expression in the head kidney and spleen after infection. In the KEGG database, we also identified that IL-17C signaling induced the expression of inflammatory cytokines IL-1β, IL-6, and IFNγ (Fig. 5A). In the large yellow croaker (Larimichthys crocea), the level of IL-17C gene expression was enhanced in the head kidney and spleen after *Aeromonas hydrophila* infection [27]. Additionally, in an *in vitro* study, recombinant IL-17C of the large yellow croaker increased the expression of IL-1β, IL-6, and IFNγ in peripheral blood leukocytes [27]. From our results and the above reports, it is suggested that IL-17C is a primary factor in the early immune response to *S. dysgalactiae* infection in cobia. The hematopoietic cell lineage (ko04640) in KEGG was significantly assigned by DEGs in head kidney 2 dpi (Table 3). We identified that neutrophil lineage-related genes (CSF3, CD121, and CD114) were upregulated in the spleen after *Streptococcus dysgalactiae* infection (Fig. 5B). Neutrophils comprise the largest proportion of peripheral leukocytes in vertebrates, and typically

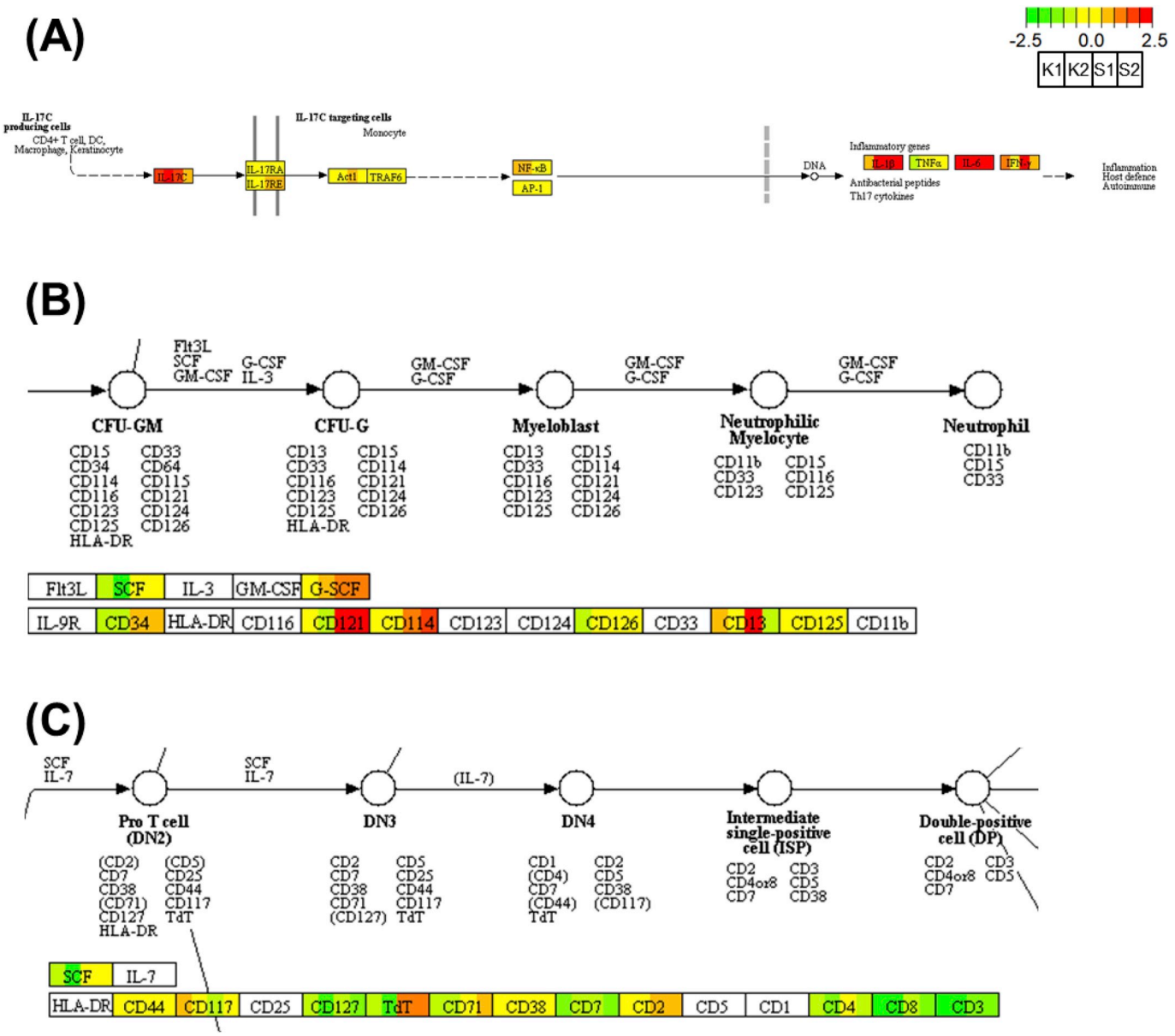

Fig. 5. Immune related pathway map in KEGG. The relative expression levels compared *Streptococcus dysgalactiae* infection group and PBS-treated group, are shown in this illustration of IL-17C pathway in IL-17 signaling pathway (ko04657), neutrophil lineage and T-cell lineage in hematopoietic cell lineage (ko04060). In each gene boxes, the gene expression levels are shown in 4 group (K, kidney; S, spleen; 1, 1 dpi; 2, 2 dpi). The lower expression levels of genes are shown in green, and the higher expression levels of genes are shown in red. Undetected genes are shown by white coloring (see color legend in figure). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

contribute to innate immunity through phagocytosis and the secretion of granule proteins [28,29]. In zebrafish, it has been demonstrated that CSF3 and its receptor, CD121, are key regulators of neutrophil production [30]. We found that many genes related to neutrophil degranulation were upregulated in the head kidney and spleen after *S. dysgalactiae* infection (Fig. 6A). Therefore, neutrophils may potentially be key to defending against *S. dysgalactiae* in cobia. In the transcriptome study of soiny mullet (*Liza haematocheila*) after *S. dysgalactiae* infection, the genes in the T-cell receptor signaling pathway were upregulated in the spleen [9]. T-cells are important lymphocytes for pathogen adaptive immunity in vertebrates [31]. Meanwhile, when studying cobia, the T-cell lineage-related genes were downregulated in the head kidney and spleen after *S. dysgalactiae* infection (Fig. 6B). Although the causes of the difference between mullet and cobia are unclear, it is possible that T-cell downregulation led to high mortality in cobia after *S. dysgalactiae* infection. Finally, we investigated the key factors for inducing the downregulation of T-cell-related genes. It has been reported that neutrophils regulate the immune system via interaction with macrophages, natural killer cells, dendritic cells, mesenchymal stem cells, B and T lymphocytes, or platelets [24]. In neutrophils and T-cells, many reports have shown that neutrophils inhibit the activation and proliferation of T-cells [32–37]. Therefore, we investigated the common DEGs in neutrophil degranulation (GO: 0043312) and negative regulation of T-cell proliferation (GO: 0042130 and GO: 0046007), based on the GO ontology database. In Fig. 6, we identified a common gene, arginase 2 (ARG2), which increased in the head kidney and spleen after *Streptococcus dysgalactiae* infection. Arginase metabolises L-arginine, which is necessary for T-cell proliferation [38], into L-ornithine and urea. In mammals, ARG1 (not ARG2) is mainly expressed in neutrophils and inhibits T-cell proliferation and activation [39,40]. We could not identify ARG1 expression in our transcriptome data (data not shown). Based on our results and the aforementioned reports, it is possible that

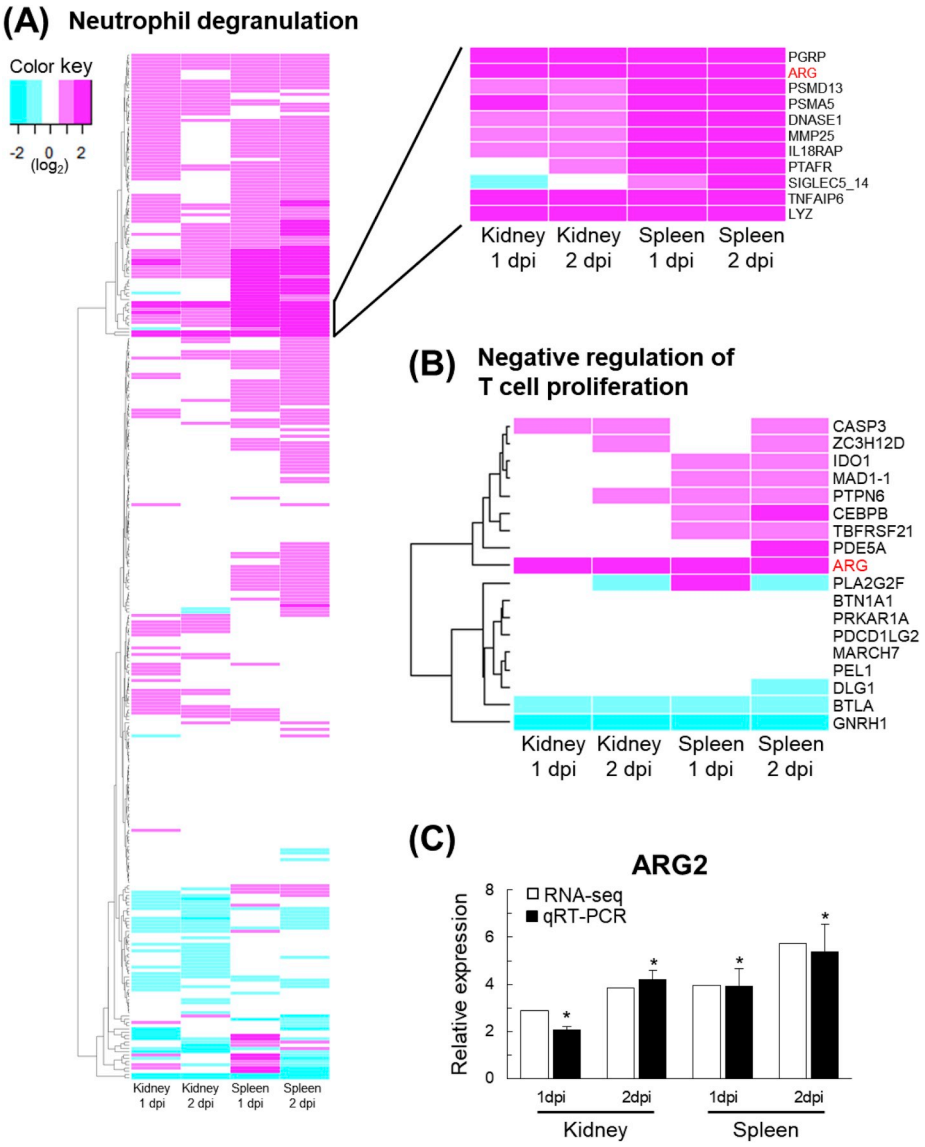

Fig. 6. ARG2 is a commonly factor in neutrophil and T-cell proliferation. Clustering by gene expression profiles related Neutrophil degranulation (A) and negative regulation of T-cell proliferation (B) in a transcriptomic dataset. Scores are colored on a log2 scale (*Streptococcus dysgalactiae* infection/PBS treatment) with magenta maximum and cyan minimum. Heat maps and clustering were generated using the gplots package of R statistical program. (C) Comparative analysis of ARG2 expression by RNA-seq and qRT-PCR. Relative expression levels was determined infection group/PBS control group. * indicates significant difference ($n = 8$, $p < 0.05$) between the infection group and PBS group. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

overexpression of ARG2 in neutrophils causes T-cell downregulation in cobia after *S. dysgalactiae* infection. In conclusion, our study provides valuable information regarding the transcriptome of cobia upon infection with *Streptococcus dysgalactiae*. In this study, we obtained a total of 106,984 unigenes, and identified immune system-related DEGs involved in the complement system, cytokine-cytokine receptor interaction, and the hematopoietic cell lineage. These pathways contribute to anti-bacterial responses after *S. dysgalactiae* infection in cobia. These data offer deeper functional analysis of the immune system and protective strategies against *S. dysgalactiae* infection.

Acknowledgements

The authors thank Genomics Bioscience Technology Co. Ltd. (Taipei, Taiwan) for assistance with transcriptome analysis. This research was funded by the National Science Council, Taiwan, grant number MOST 107-2313-B-020 -012 -MY3

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.07.009.

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