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Comparative transcriptome analysis of the transcriptional heterogeneity in different IgM$^+$ cell subsets from peripheral blood of Nile tilapia (*Oreochromis niloticus*)

Liting Wu, Yanjian Yang, Linghe Kong, Xia Bian, Zheng Guo, Shengli Fu, Fang Liang, Bingxi Li, Jianmin Ye*

Institute of Modern Aquaculture Science and Engineering, School of Life Sciences, South China Normal University, Guangdong Provincial Key Laboratory for Healthy and Safe Aquaculture, Guangdong Provincial Engineering Technology Research, Center for Environmentally-Friendly Aquaculture, Guangzhou, 510631, PR China

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ABSTRACT

In teleost fish, IgM$^+$ B cells play important roles in innate and adaptive immunity. Different IgM$^+$ B cells are detected in teleost, named IgM^{lo} and IgM^{hi} B cell subsets, according to the distinct expression levels of membrane IgM (mIgM). However, the study on the heterogeneity in IgM$^+$ B cell subsets remains poorly understood. In this study, the comparative transcriptomic profiles of IgM$^-$, IgM^{lo} and IgM^{hi} from peripheral blood of Nile tilapia (*Oreochromis niloticus*) were carried out by using RNA-sequencing technique. A total of 6045 and 5470 differentially expressed genes (DEGs) were detected in IgM^{lo} and IgM^{hi} cells, respectively, as compared with IgM$^-$ lymphocytes, whereas 3835 genes were differentially expressed when IgM^{lo} compared to IgM^{hi} cells. Analysis of the KEGG database indicated that the DEGs were enriched in immune system categories and signaling transduction and interaction in IgM$^-$ vs IgM^{hi}, IgM$^-$ vs IgM^{lo} and IgM^{lo} vs IgM^{hi}. Comparatively, in IgM^{lo} vs IgM^{hi}, GO enrichment analysis indicated that the DEGs enriched in nucleic acid binding transcription factor activity. Analysis of crucial transcription factors for B cell differentiation indicated that IgM^{lo} and IgM^{hi} cell clusters belonged to the different B cell subsets. The data generated in this study may provide insights into understanding the heterogeneity of IgM$^+$ cells in teleost, and suggest that IgM$^+$ B cells play a crucial role in innate immunity.

1. Introduction

Teleost fish B cells are key players in adaptive immune responses through the production of antibodies [1–3]. B cells produce antibodies (immunoglobulins, Igs) to recognize microbial antigens, neutralize their infectivity and help in their elimination [4]. The Igs in teleost include three heavy Ig chain classes named IgM, IgT/Z, and IgD as defined by the expressed genes μ, τ, and δ, respectively [5–8]. Two different groups of B cells have been identified in rainbow trout (*Oncorhynchus mykiss*), IgM$^+$/IgD$^+$/IgT$^-$ (IgM$^+$ cells) and IgM$^-$/IgD$^-$/IgT$^+$ (IgT$^+$ cells) [8,9]. Whereas in catfish (*Ictalurus punctatus*) IgD$^+$/IgM$^-$ populations have also been described instead of IgT$^+$ cells since IgT has not been found thus far in this fish [10]. Among them, the mRNA transcripts for IgM are the highest [8], and the IgM is to be the main Ig secreted in plasma [11,12]. Membrane IgM$^+$ (mIgM$^+$) B cells, the majority B cells in teleost, have been demonstrated in a variety of fish species, such as rainbow trout, channel catfish, lumpfish (*Cyclopterus lumpus* L.), half-smooth tongue sole (*Cynoglossus semilaevis*), Atlantic salmon (*Salmo salar* L.), Atlantic cod (*Gadus morhua* L.), turbot (*Scophthalmus maximus*) and Japanese flounder (*Paralichthys olivaceus*) [13–20], which exist in different tissues but mainly in immune tissues, such as anterior kidney, spleen and peripheral blood [19]. Anterior kidney, the main hematopoietic organ, is a main site for B lymphogenesis throughout life [21–23]. It is also thought to behave as a secondary organ with developing B cells and Ig-secreting lymphocyte populations but few resting, mature B cells [24,25]. The spleen is the main secondary lymphoid tissue in teleost as in mammals, which appears to be an important site for B cell activation and plasmablast formation [26]. Peripheral blood leukocytes (PBL) contribute for more than 30–40% of the B cells with most resting, mature B cells [25,27]. Distribution analysis of mIgM$^+$ cell in immune tissues by flow cytometry revealed that the maximum mIgM$^+$ in PBL, followed by spleen and anterior kidney in rainbow trout [16,19]. Recently, a study on IgM$^+$ B cell heterogeneity in trout was characterized and demonstrated that IgM$^+$ B cells could

* Corresponding author. School of Life Sciences, South China Normal University, No.55 West Zhongshan Avenue, Guangzhou, Guangdong Province, 510631, PR China.
E-mail address: jmye@m.scnu.edu.cn (J. Ye).

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play an important role in innate immunity through constitutive expression of the related Toll-like receptor (TLR) genes [19]. However, besides the TLRs, the other molecules or factors involving the role of the IgM^+ B cells in innate immunity needs further exploration in teleost.

There had been proved that the main developmental stages of B lymphoid lineage in teleost were well-conserved with other vertebrate species [28]. The earliest stages are the common lymphoid progenitor, pro-B and pre-B cell stages, followed by immature and mature B cell stages. The approach to dissect teleost B cell development is to use nuclear transcription factors that are differentially expressed during B cell development [25,28–30]. During B cell activation, the reduced expression of paired box 5 (Pax5) level is partly caused by induction of the transcriptional repressor lymphocyte-induced maturation protein-1 (Blimp1) [31–33]. Blimp1 in turn shifts Ig expression from the membrane to the secreted form, which leads to increases in secreted Ig expression in activated B cells with simultaneous reduction of membrane Ig [34]. Mature B cells express high membrane Ig, activated B cells and plasmablasts express lower and plasma cells lack membrane Ig [35,36]. Based on the expression of membrane IgM, Pax5 and Blimp1, different IgM^+ B cell subsets residing within the peritoneal cavity of vaccinated rainbow trout were studied and resembled IgM^{hi} and IgM^{lo} B cells as naïve B cells and antibody-secreting cells, respectively [20]. These studies sheds light on the heterogeneity of B cell subsets. Moreover, the analysis of the role of other molecules or transcription factors on B cell differentiation will provide insights into understanding of the heterogeneity of the IgM^+ B cell subsets.

In the absence of the monoclonal antibody (mAb) of special membrane surface protein except mIg, studies on the heterogeneity of molecular mechanism of teleost B cell subsets still remains elusive. RNA sequencing (RNA-Seq) helps to identify and quantify transcripts without prior knowledge of a specific gene, obtaining sequence variation information and alternative splicing [37]. Monoclonal antibody of mIgM and RNA-seq technique provide us methods to explore this challenge [28].

Nile tilapia (*Oreochromis niloticus*) is one of main aquaculture species having high economic value and China is the largest tilapia producer in the world [38,39]. Studies on the immune system in tilapia are popular in recent years and are important for basic immunological studies [40–45]. Different from the rainbow trout, there are only few studies on tilapia IgM^+ B cell subset heterogeneity. In this study, we used RNA-Seq for comparative transcriptome profile analysis of the heterogeneity in IgM^+ cell subsets from PBL of Nile tilapia, including IgM^-, IgM^{lo} and IgM^{hi} lymphocytes. The information of transcriptome sequences and differentially-expressed genes (DEGs) related to innate immunity may contribute to further understanding of B cell activity and the characterization of different B cell subsets in teleost fish.

2. Materials and methods

2.1. Experimental fish

Nile tilapia were cultured in Guangdong Tilapia Breeding Farm (Guangdong, China), and kept a weight at 500 ± 50 *g*. The fish were acclimated in the automatic filtering aquaculture system with a stocking rate of 10 g/L under 28 ± 2 °C for three weeks [42,46,47]. All animal protocols were reviewed and approved by the University Animal Care and Use Committee of the South China Normal University.

2.2. Cell sorting of peripheral blood (PBL)

Prior to bleeding, fish were anesthetized in water containing 0.2 g/L MS-222 (Aladdin, China). Blood from three fish was individually collected by venipuncture from the caudal vessel with 100 μL sterile heparin (0.1 g/L) in 1 mL syringe, and then placed in a sterile 1.5 mL tube [2]. After centrifugation at 500 × *g*, 4 °C for 10 min, the serum was removed. The precipitated blood was diluted 4 times with RPMI-1640 (Gibco, USA), supplemented with 100 U/mL penicillin G (Sigma, USA), 100 μg/mL streptomycin (Sigma, USA), 10 units/mL heparin (Sigma, USA) [48]. The leukocytes were isolated via Histopaque 1077 (Sigma, USA) density centrifugation at 4 °C, 500 × *g* for 40 min [26]. The interface cells were collected and washed twice with RPMI-1640 containing 5% fetal bovine serum (FBS; Gibco, USA). After cell counting using trypan blue (excluded few died cells were dyed to blue), the cells were adjusted to the concentration at 1×10^7 cells/mL. The purified leukocytes were incubated with anti-IgM mouse mAb [42,46] (1 mg/mL, 1:2000 dilution) labeled by Alexa Fluor 647 (AF647; Thermo, USA) at room temperature for an hour. Following two washing steps, cells were re-suspended in RPMI-1640 contained 5% FBS and IgM^+ cells were sorted with a BD FACS Aria III flow cytometer (BD, USA). Using first their forward scatter (FSC)/side scatter (SSC) profiles (to exclude the granulocyte gate [20]) and then on the basis of the fluorescence emitted by the sample. The three fractions, IgM^-, IgM^{hi} and IgM^{lo} cells from individual fish were then collected in different tubes. After sorting, every fraction was sorted back to test the purity and stored in liquid nitrogen until use.

2.3. RNA extraction, library construction and sequencing

Total RNAs of each sample were prepared using Trizol reagent (Vazyme, China) according to the manufacturer's protocol. Each sample from three fish was individually prepared for RNA extraction. The quality and quantity of RNA samples were examined by use of the Agilent 2100 Bioanalyzer (Agilent Technologies, USA) and the integrity was assessed by electrophoresis on 0.8% RNase-free agarose gel. The optical density of RNA was checked with NanoDrop 2000 (Thermo, USA). RNA samples with high purity (260/280 nm ratios between 1.8 and 2.0) and high integrity (RIN > 8.0) were used for subsequent analyses. The cDNA library construction and sequencing of three RNA samples were sequenced using Illumina HiSeq™ 2000 by Gene Denovo Biotechnology Co. Ltd (Guangdong, China).

2.4. Sequence alignment and functional annotation

The high-quality clean reads were obtained from raw reads after removal of adapters, reads with more than 10% unknown bases and reads with more than 50% low-quality bases, in accordance with the criteria of low-quality bases with sequencing quality no higher than 5. All clean reads were then well mapped to the Nile tilapia genome (NCBI, *Oreochromis niloticus*, ASM185804v2) using TopHat2 software [49] and only unique mapping reads were retained for calculating gene expression. In order to identify new genes and new splice variants of known genes, the transcripts were reconstructed using Cufflinks (version 2.2.1) [50] based on reference annotation-based transcripts. Cufflinks constructed faux reads according to references to compensate for the influence of low coverage sequencing. During the last assembly step, all of the reassembled fragments were aligned with reference genes and similar fragments were then removed. All of the transcripts including new gene transcripts (≥200 bp and exon number > 2) were annotated using the BLASTx function with protein databases for functional annotation, including the NCBI Nr protein database (https://ftp.ncbi.nlm.nih.gov/blast/db/FASTA/), Gene ontology (GO) (http://www.geneontology.org/), and Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.genome.jp/kegg/kegg2.html) databases with a significance threshold of *E* value < 10^{-5}. GO annotation was conducted using Blast2GO (vision 2.5) software [51] and Web Gene Ontology Annotation Plot (WEGO) software (version 2.0) was then applied for gain GO function classification [52]. The KEGG Automatic Annotation Server (KAAS) (http://www.genome.jp/tools/kaas/) was used to conduct KEGG pathway analyses.

2.5. Differentially expressed genes (DEGs) screening

The method based on fragments per kilobase of exon per million mapped reads (FPKM) is used to calculate gene expression, and the equation used was FPKM = 10^9 (C)/NL. The given FPKM (A) is the expression of gene A, C is the number of reads uniquely aligned to gene A, N is the total number of reads uniquely aligned to all genes, and L is the number of bases in gene A.

Poisson distribution analysis was performed to screen the DEGs identified by the edge package (http://www.r-project.org/). To correct for multiple testing, the false discovery rate (FDR) was calculated to adjust the threshold of *p* value [53]. The significance of the difference in gene expression was calculated when FDR was < 0.05 and the values of FPKM showed at least a 2-fold difference in expression (fold change, FC) ($|\log_2 FC| \geq 1$) [54]. Hierarchical clustering of the DEGs was performed using the OmicShare tools, which is a free online platform for data analysis (http://www.omicshare.com/tools).

The DEGs were then annotated with the COG database to predict and classify possible functions using BLASTx (*E* value < 10^{-5}). GO enrichment analysis of DEGs was implemented using the GOseq R package (Bioconductor version: release (3.7)) [50]. The enrichment of the DEGs in KEGG pathways was tested using the KOBAS software (version 2.0) [55]. GO terms and KEGG pathways with corrected Q values < 0.05 (Q value is a corrected *p* value after multiple hypothesis tests) were considered significantly enriched for DEGs.

2.6. Gene expression validation with real-time quantitative PCR (RT-qPCR)

In order to verify the reliability of RNA-seq, 12 DEGs among IgM^{hi}, IgM^{lo} and IgM^- groups were selected for RT-qPCR assay, including B-cell antigen receptor complex-associated protein alpha and beta chains (CD79a, CD79b), B-cell linker (BLNK), tyrosine-protein kinase Lyn (LYN), Pax5, Blimp1, DNA-binding protein 1 (Ikaros1), transcription factor COE1 (EBF1), C–C chemokine receptor type 7 (CCR7), C-X-C chemokine receptor type 4 (CXCR4), interleukin-10 (IL-10) and interleukin-6-like (IL-6). Specific primers were designed using Primer premier 5 software and synthesized by TSINGKE Biotech Co., Ltd (TSINGKE, China). The primer sequences were listed in Table S1. The housekeeping gene *β*-actin was used as the reference gene. The RT-qPCR was performed on ABI QuantStudio™ 5 RT-qPCR system (Thermo, USA) using a SYBR Green-based PCR assay. Each reaction mixture was 20 μL, containing 3 μL of diluted first-strand cDNAs, 3 μL DEPC water, 250 nM of each primer 2 μL, and 10 μL of SYBR Green PCR Master Mix (Yeasen, China). All reactions were performed in triplicates and the RT-qPCRs were run the fast mode as follows: 95 °C for 20 s, followed by 40 cycles of 95 °C for 10 s and 60 °C for 20 s in 96-well optical reaction plates. Dissociation curve analysis was performed to determine the target specificity and the program are 95 °C for 1 s and 60 °C for 20 s. The relative expression ratio of the target genes versus *β*-actin [42,46] gene was calculated using $2^{-\triangle CT}$ method and all data were given in terms of relative mRNA expression. The transcriptional level of membrane IgM (mIgM) was detected by RT-qPCR in different samples before the samples were delivered to transcriptome analysis. The primers of mIgM refer to our published work [42]. A regression analysis was performed between RT-qPCR and RNA sequencing including all genes in these three samples using SigmaPlot 12.5 software. The total RNA from each individual fish was as a sample, and three fish were individually performed for RT-qPCR analysis.

2.7. Key resources table

Resource	Source	Identifier
Antibodies		

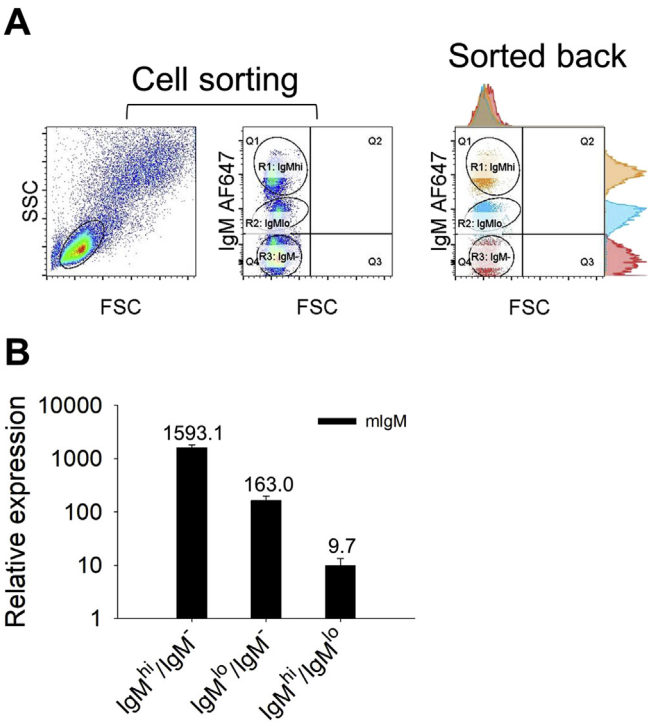

Fig. 1. Cell sorting and post sort analysis. (A) Left panel: Scatterplot of a representative PBL sample. The oval gate defined the live single lymphocytes. Middle panel: Scatterplot showing different IgM^+ (AF647), including IgM^{hi} (the R1 gate), IgM^{lo} (the R2 gate) and the IgM^- was shown at the R3 gate. Right panel: The merge of three plots show the post sorted analysis of IgM^-, IgM^{lo} and IgM^{hi} cells. (B) Ratio of mIgM transcription in IgM^{hi} cells, IgM^{lo} cells and IgM^- cells in comparison to each other levels.

Table 1
Major characteristics of mRNA libraries and assembly statistics for the three-transcriptome data of *Oreochromis niloticus*.

Groups	IgM^-	IgM^{lo}	IgM^{hi}
Clean reads No.$^{\alpha}$ (× 10^6)	50.67	68.08	62.58
HQ$^{\alpha}$ clean reads No. (× 10^6)	49.77 (98.25%)	66.89 (98.25%)	61.43 (98.15%)
Adapters No. (× 10^6)	0.2 (0.4%)	0.27 (0.39%)	0.25 (0.4%)
LQ$^{\alpha}$ reads No. (× 10^6)	0.68 (1.35%)	0.92 (1.35%)	0.90 (1.44%)
Q20 (%)	98.9	98.91	98.89
GC (%)	48.28	49.00	48.54
Total reads No. (× 10^6)	49.48	66.39	61.16
Unmapped reads (× 10^6)	6.86 (13.87%)	8.51 (12.82%)	8.33 (13.63%)
Unique mapped reads (× 10^6)	41.54 (83.94%)	56.07 (84.45%)	51.82 (84.72%)
Multiple mapped reads (× 10^6)	1.08 (2.18%)	1.81 (2.72%)	1.01 (1.65%)
Mapping ratio	86.13%	87.18%	86.37%

α: No. is short for number; HQ and LQ represent high quality and low quality, respectively.

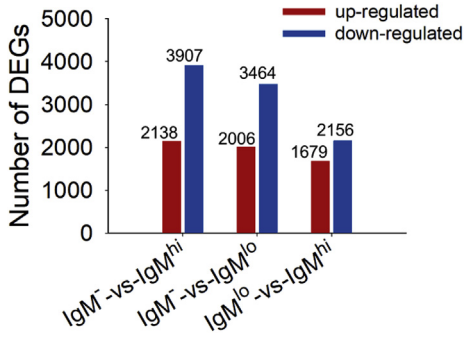

Fig. 2. Pairwise comparison of differentially DEGs among the IgM^{-}, IgM^{lo} and IgM^{hi} lymphocytes from PBL in Nile tilapia.

anti-IgM mouse mAb	The anti-OnIgM mAb was prepared by Guangdong Provincial Key Laboratory for Healthy and Safe Aquaculture, South China Normal University. It had been used to perform the study in Nile tilapia and the results had been published [1–3]. [1] X. Bian, L. Wu, L. Mu, X. Yin, X. Wei, X. Zhong, Y. Yang, J. Wang, Y. Li, Z. Guo, J. Ye, Spleen tyrosine kinase from Nile tilapia (Oreochromis niloticus): Molecular characterization, expression pattern upon bacterial infection and the potential role in BCR signaling and inflammatory response, Fish Shellfish Immunol 82 (2018) 162–172 [2]. X. Wei, B. Li, L. Wu, X. Yin, X. Zhong, Y. Li, Y. Wang, Z. Guo, J. Ye, Interleukin-6 gets involved in response to bacterial infection and promotes antibody production in Nile tilapia (Oreochromis niloticus), Dev Comp Immunol 89 (2018) 141–151 [3]. L. Wu, X. Bian, L. Kong, X. Yin, L. Mu, S. Wu, A. Gao, X. Wei, Z. Guo, J. Ye, B cell receptor accessory molecule CD79 gets involved in response against Streptococcus agalactiae infection and BCR signaling in Nile tilapia (Oreochromis niloticus), Fish Shellfish Immunol 87 (2019) 212–219.	
Chemical		
DEPC	Sigma	150-38-9
Histopaque	Sigma	Cat#10771
MS-222	Aladdin	886-86-2
penicillin G	Sigma	P0781
streptomycin	Sigma	P0781

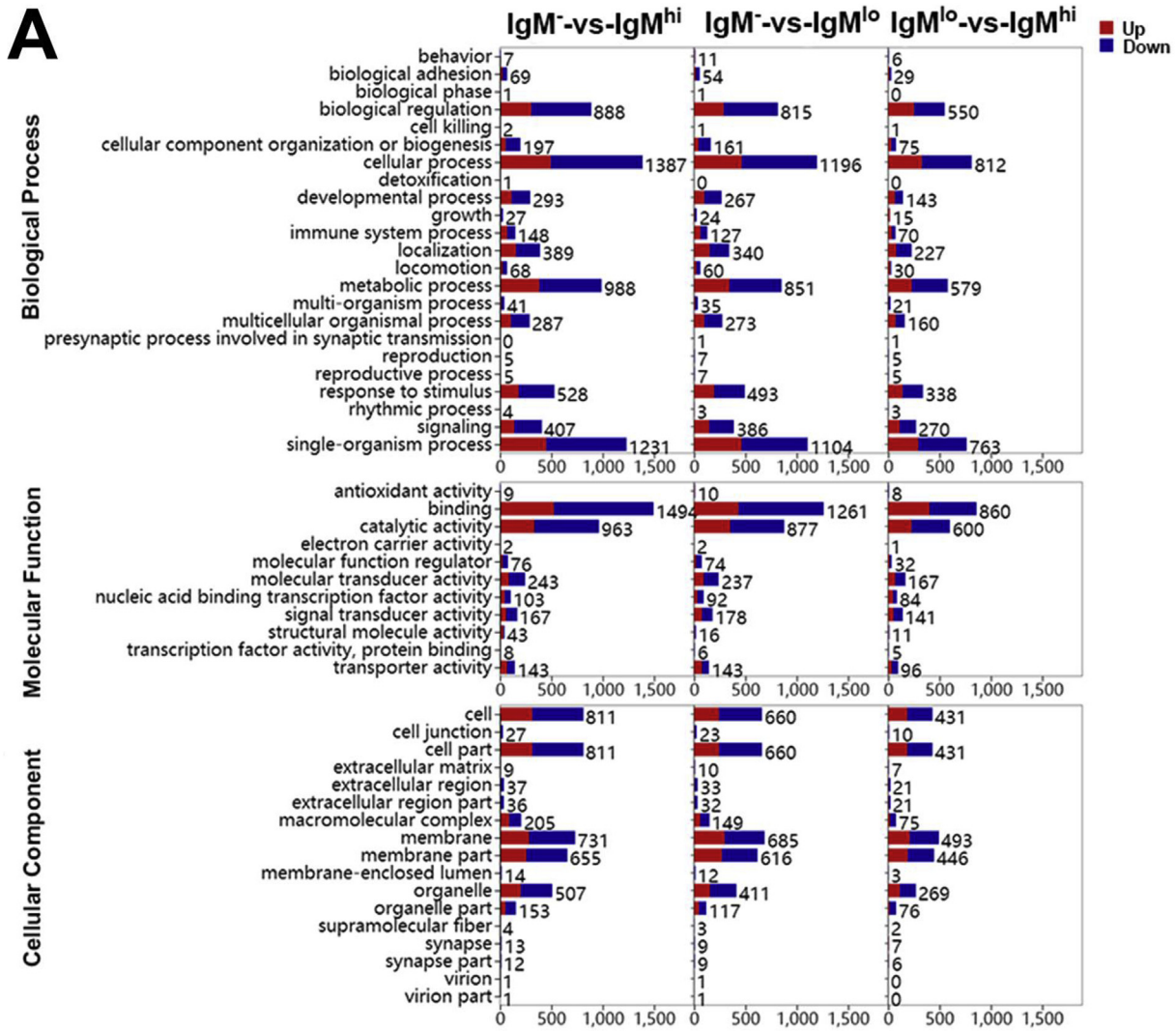

Fig. 3. GO distribution (A) and the heatmap of GO enrichment analysis (B) for the DEGs in IgM^{-} vs IgM^{hi}, IgM^{-} vs IgM^{lo} and IgM^{lo} vs IgM^{hi}. The significance of the most represented GO terms in each main GO ontology is indicated by Q value (< 0.05). The red areas represented the significant Q values, whereas the green represented the nonsignificant values. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

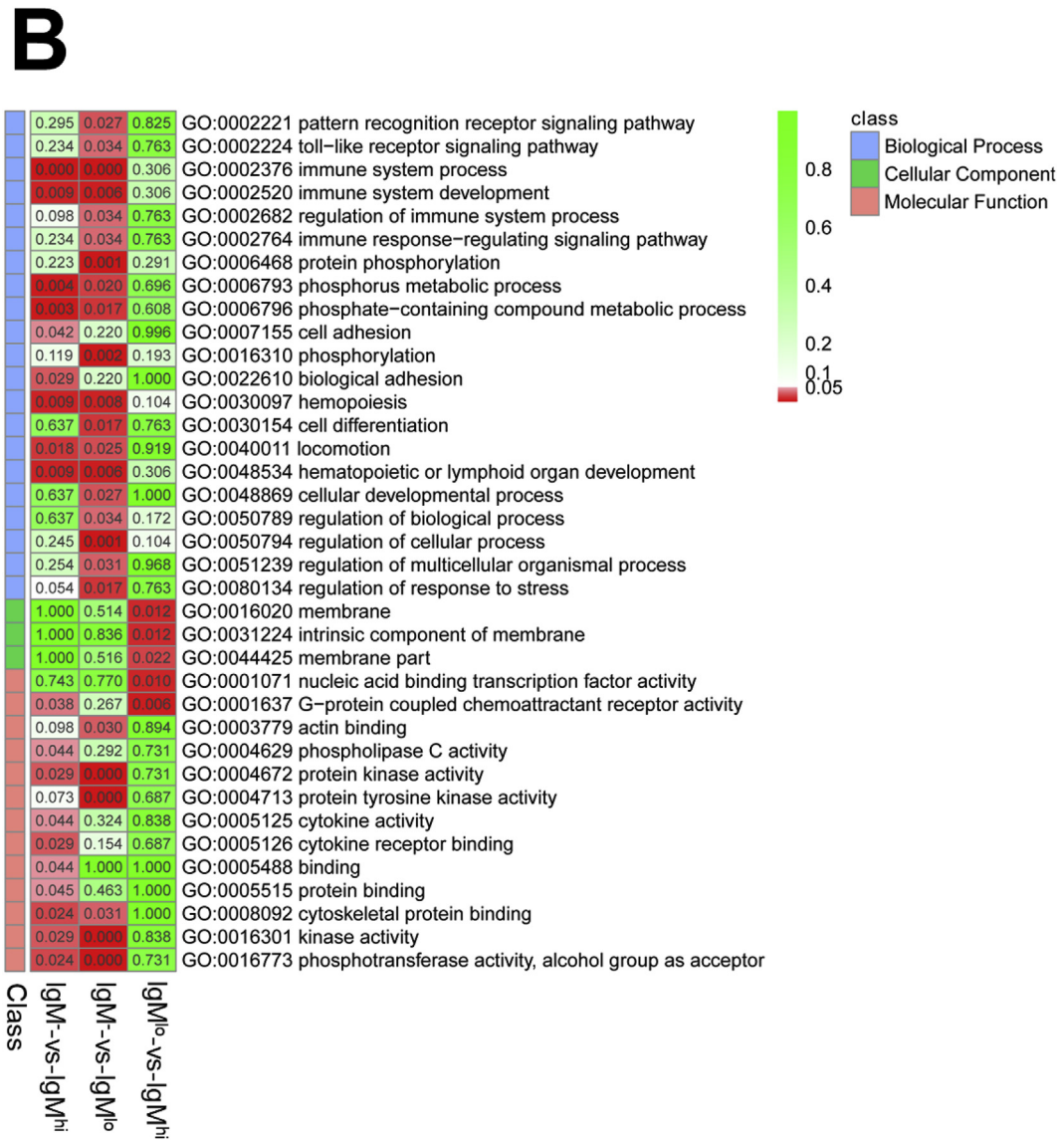

Fig. 3. (*continued*)

trypan blue	Sigma	Cat#T6146

3. Results

3.1. Identification of IgM$^+$ B cell populations in PBL

Prior to establish the differentiation of PBL B cell subsets, a phenotypic characterization of the IgM$^+$ B cells residing in PBL of healthy fish was performed. To carry this out, leukocytes from PBL were isolated and labeled with anti-IgM mAb [42,46]. In order to analyze B cells, the FSC^{lo} SSC^{lo} population was gated and named lymphoid gate, which excludes granulocytes and myeloid cells [20] (Fig. 1A, left panel). It was obvious that there are two IgM$^+$ B cell subsets with different fluorescence intensity (Fig. 1A, middle panel), named IgM^{hi} and IgM^{lo} B cells. To further characterize these two IgM$^+$ B cell populations, IgM^{hi} and IgM^{lo} cells were isolated by flow cytometry. For the purpose of comparison, IgM$^-$ cells were isolated as well (Fig. 1A, middle panel). The purity of the isolated populations was analyzed after sorting and the purity level shown that it was higher than 95%, which demonstrated the sorting effectiveness (Fig. 1A, right panel). The expression of mIgM in different IgM$^+$ cells relative to the expression of β-actin was studied and the ratio of each other was shown in Fig. 1B, which suggested that the IgM^{hi} B cells express the highest level of mIgM (1593.1-fold compared to IgM$^-$). The IgM^{lo} B cells was the second-high expression of mIgM (163-fold compared to IgM$^-$).

3.2. Sequence analysis of IgM$^-$, IgM^{lo} and IgM^{hi} lymphocytes

The raw data have been submitted to the Sequence Read Archive (SRA) database, and the ID number of the database is PRJNA558099. To compare the gene expression profiles in IgM$^-$, IgM^{lo} and IgM^{hi} lymphocytes, cDNA libraries were constructed from healthy Nile tilapia PBL, which were then sequenced using the Illumina HiSeq™ 2000 platform (Illumina, USA). After cleaning and checking the read quality, approximately 50.67–68.08 million clean reads were generated for the three samples through RNA sequencing, where the clean data GC content ranged from 48.28% to 49.00%, and the Q20% exceeded 98.89% (Table 1). The high-quality clean reads were then aligned with Nile tilapia genome (assembly ASM185804v2) sequences in the NCBI database (https://www.ncbi.nlm.nih.gov/genome/?term=ASM185804v2), where the mapped reads efficiency ranged from 86.13% to 87.18% (Table 1). Thus, the throughput and sequencing quality were sufficiently high to warrant further analysis in the present study.

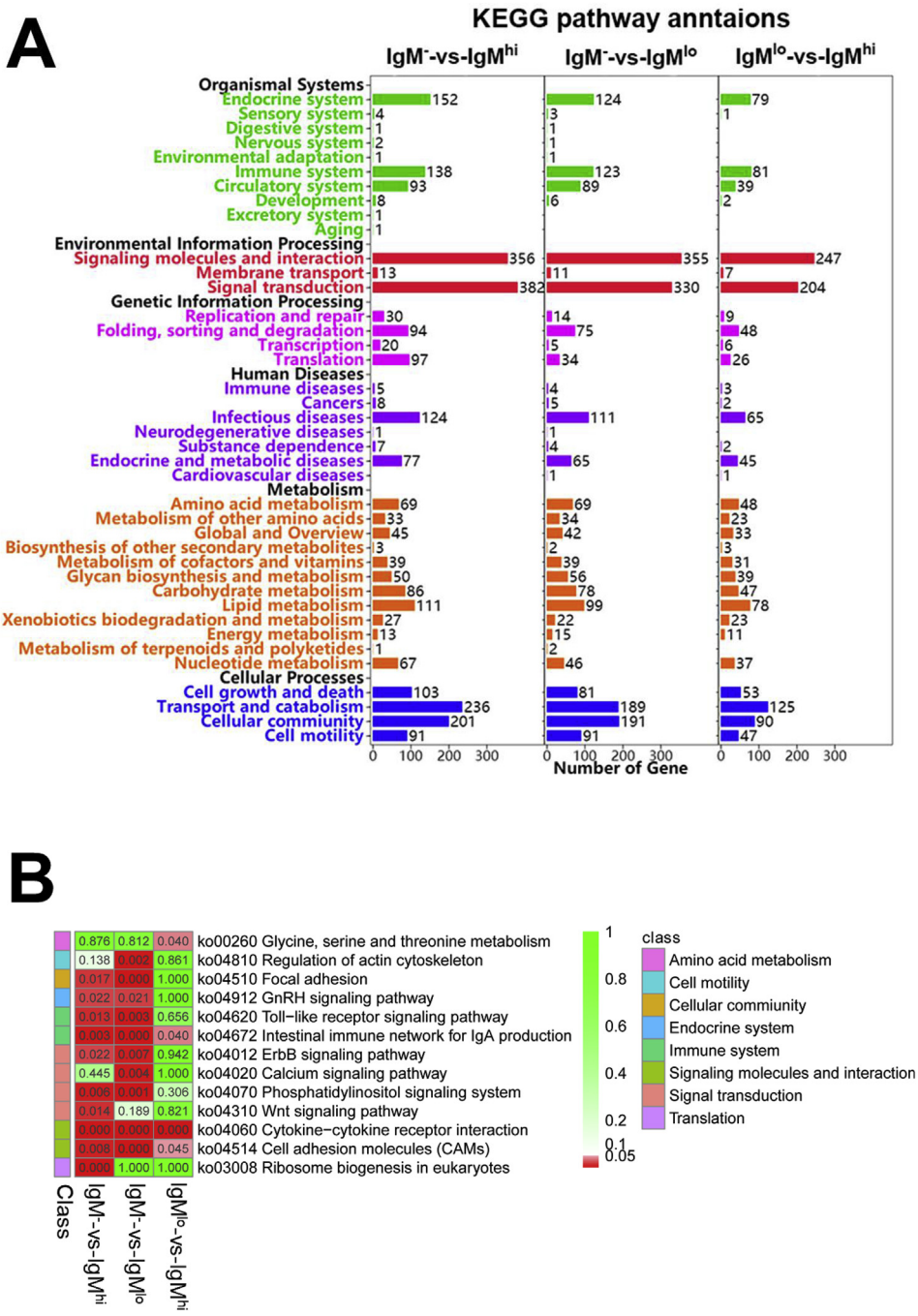

Fig. 4. Distribution of DEGs in IgM^{-} vs IgM^{hi}, IgM^{-} vs IgM^{lo} and IgM^{lo} vs IgM^{hi} in KEGG pathway analysis (A) and the heatmap of KEGG enrichment pathway in each main cluster (B) (Q value < 0.05). KEGG enrichment analysis of DEGs was classified into six categories: cellular processes, environmental information processing, genetic information processing, human diseases, metabolism, and organismal systems. The X-axis identifies the name of the gene functional classification in KEGG, and the Y-axis shows the numbers of annotated genes. The red areas represented the significant Q values, whereas the green represented the non-significant values. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 2
Analysis of immune system-related pathways.

Pathway ID	Pathway	Number of DEGs with pathway annotation		
		IgM^{-} vs IgM^{hi} (1572)	IgM^{-} vs IgM^{lo} (1350)	IgM^{lo} vs IgM^{hi} (722)
ko04672	Intestinal immune network for IgT production	50 (3.18%)	54 (4%)	31 (3.36%)
ko04620	Toll-like receptor signaling pathway	54 (3.44%)	50 (3.7%)	27 (2.93%)
ko04621	NOD-like receptor signaling pathway	36 (2.29%)	25 (1.85%)	24 (2.6%)
ko04622	RIG-I-like receptor signaling pathway	23 (1.46%)	8 (0.59%)	8 (0.87%)
ko04623	Cytosolic DNA-sensing pathway	22 (1.4%)	17 (1.26%)	11 (1.19%)
ko04660	T cell receptor signaling pathway	1 (0.06%)	1 (0.07%)	1 (0.11%)
ko04062	Chemokine signaling pathway	2 (0.13%)	1 (0.07%)	0
ko04650	Natural killer cell mediated cytotoxicity	2 (0.13%)	1 (0.07%)	0
ko04662	B cell receptor signaling pathway	1 (0.06%)	0	0
ko04664	Fc epsilon RI signaling pathway	1 (0.06%)	0	0

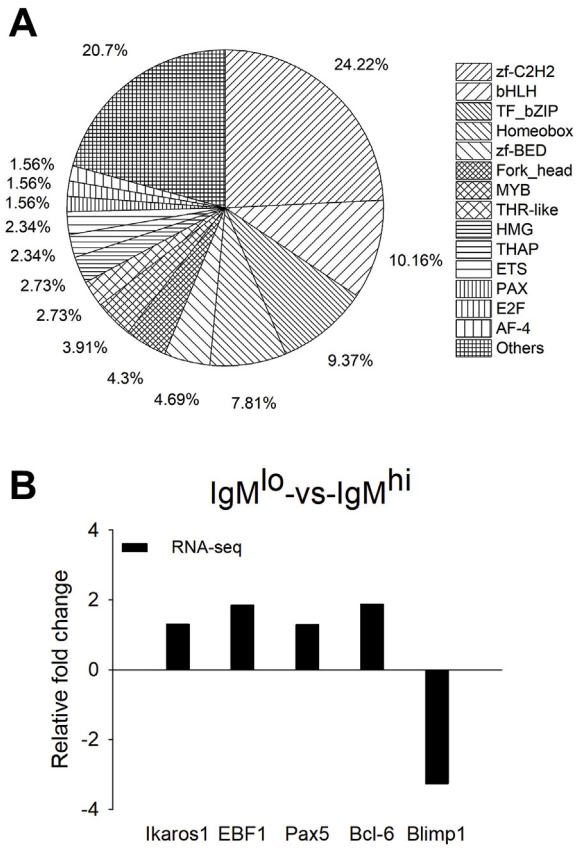

Fig. 5. Percentages of different transcription factors involved in IgM^{lo} vs IgM^{hi} group (A) and the expression profiles of B cell development related transcription factors (B).

3.3. Identification of DEGs in IgM^{-}, IgM^{lo} and IgM^{hi} lymphocytes

The FPKM method was used to analyze the gene expression. By remapping to the reference genome, 20,758, 20,975 and 20,722 expressed genes were identified in IgM^{-}, IgM^{lo} and IgM^{hi} lymphocytes, respectively. Using a cutoff of 2-fold change ($|\log_2 FC| > 1$ and false discovery rate (FDR) < 0.05 in IgM^{-}, IgM^{lo} and IgM^{hi} lymphocytes as methodological description by pairwise comparison, a total of 6045 (2138 genes upregulated and 3907 genes downregulated) and 5470 (2006 genes upregulated and 3464 genes downregulated) DEGs were detected in IgM^{hi} and IgM^{lo} B cells, respectively, as compared with IgM^{-} lymphocytes, whereas 3835 genes (1679 genes upregulated and 2156 genes downregulated) were differentially expressed in IgM^{lo} lymphocytes when comparing with IgM^{hi} B cells (Fig. 2).

3.4. GO analysis of DEGs

In order to understand the potential functions of the DEGs, we determined them in terms of biological process, molecular function and cellular component according to the GO database (Fig. 3, Tables S2–S4). In IgM^{-} vs IgM^{hi}, 5961 DEGs were assigned to many GO terms in categories of the biological process (2246 DEGs, 37.7%), molecular function (2271 DEGs, 38.1%), and cellular component (1444 DEGs, 24.2%) (Fig. 3A, Table S2). Meanwhile, in IgM^{-} vs IgM^{lo}, 5243 DEGs were assigned to many GO terms in the biological process (1966 DEGs, 37.5%), molecular function (2010 DEGs, 38.3%), and cellular component (1267 DEGs, 24.2%) (Fig. 3A, Table S3). In IgM^{lo} vs IgM^{hi}, 3558 DEGs were assigned to GO terms in the biological process (1320 DEGs, 37.1%), molecular function (1375 DEGs, 38.6%), and cellular component (863 DEGs, 24.3%) (Fig. 3A, Table S4).

The significant annotated GO biological process pathway in IgM^{-} vs IgM^{hi} and IgM^{-} vs IgM^{lo} was varied from each other, but the same top significant one was immune system process (GO:0002376) with 148 (6.59%) and 128 (6.51%) DEGs, respectively (Fig. 3B, Tables S2–S3). However, there was no significant annotated GO biological process pathway in IgM^{lo} vs IgM^{hi} (Fig. 3B, Table S4). In molecular function category, the top significant annotated term in IgM^{-} vs IgM^{hi} and IgM^{-} vs IgM^{lo} was cytoskeletal protein binding (73, 3.21%) and protein tyrosine kinase activity (41, 2.04%), respectively; whereas in IgM^{lo} vs IgM^{hi} was G-protein coupled chemoattractant receptor activity (21, 1.53%) (Fig. 3B, Tables S2–S4). In cellular component ontology, there was no significant enriched term in IgM^{-} vs IgM^{hi} and IgM^{-} vs IgM^{lo}, while there were three terms, including membrane (494, 57.24%), intrinsic component of membrane (426, 49.36%) and membrane part (447, 51.8%) in IgM^{lo} vs IgM^{hi} (Fig. 3B, Table S4).

3.5. KEGG pathway of DEGs

On the basis of these DEG findings, we performed KEGG pathway classification for DEGs (Fig. 4, Tables S5–S7). DEGs were annotated into 6 main categories, including cellular processes, environmental information processing, genetic information processing, metabolism, human diseases, and metabolism organismal systems. Overall, for each group, the top two frequently annotated DEGs under the environmental information processing category were signal molecular and interaction and signal transduction (Fig. 4A).

In IgM^{-} vs IgM^{hi}, 10 KEGG pathways were significantly (a cut-off FDR corrected p value, Q value < 0.05) assigned to DEGs (Fig. 4B), which included several immune-related pathways, such as Intestinal immune network for IgT production (50, 3.18%) and Toll-like receptor signaling pathway (54, 3.44%) (Table S5). In IgM^{-} vs IgM^{lo}, 10 KEGG pathways were significantly (Q value < 0.05) assigned to DEGs (Fig. 4B) and immune-related pathways included Intestinal immune network for IgT production (54, 4%) and Toll-like receptor signaling pathway (50, 3.7%) (Table S6). In IgM^{lo} vs IgM^{hi}, 4 KEGG pathways were significantly (Q value < 0.05) assigned to DEGs (Fig. 4B), which included Cytokine-cytokine receptor interaction (82, 8.89%), Glycine, serine and threonine metabolism (16, 1.74%), Intestinal immune network for IgT production (31, 3.36%) and Cell adhesion molecules (CAMs) (74, 8.03%) (Table S7).

3.6. The immune-related DEGs and pathway analyses

Based on the KEGG pathway annotations, 10 pathways related to the immune system were found in the present study (Table 2). Among them, Intestinal immune network for IgT production and Toll-like receptor signaling pathway had most of the genes. Other immune pathways with high number of genes included NOD-like receptor signaling pathway and RIG-I-like receptor signaling pathway (Table 2). DEGs involved in these immune pathways in different groups were listed in Tables S8–S10. And numbers of key representational immune-associated DEGs in the pathway of immune system category were showed in Table S16 including the representational TLR2, TLR3, CXCR4, IL-6 and IL-10 *etc*. DEGs in these three groups in signal transduction and signaling molecules and interaction categories were analyzed as listed in Tables S11–S13 and Table S15. DEGs that significantly related to immune response in these signaling pathways were selected and showed in Table S16 as well.

3.7. Analysis of transcription factors (TFs) in IgM^{lo} vs IgM^{hi}

Transcription factors are important regulators that can active or repress the expression of genes in a sequence specific manner, thereby affecting many biological processes. We found that the GO term "nucleic acid binding transcription factor activity" (GO: 0001071) was significant enriched in the molecular function in IgM^{lo} vs IgM^{hi} (Fig. 3B). According to the statistics, in IgM^{lo} vs IgM^{hi}, 256 DEGs

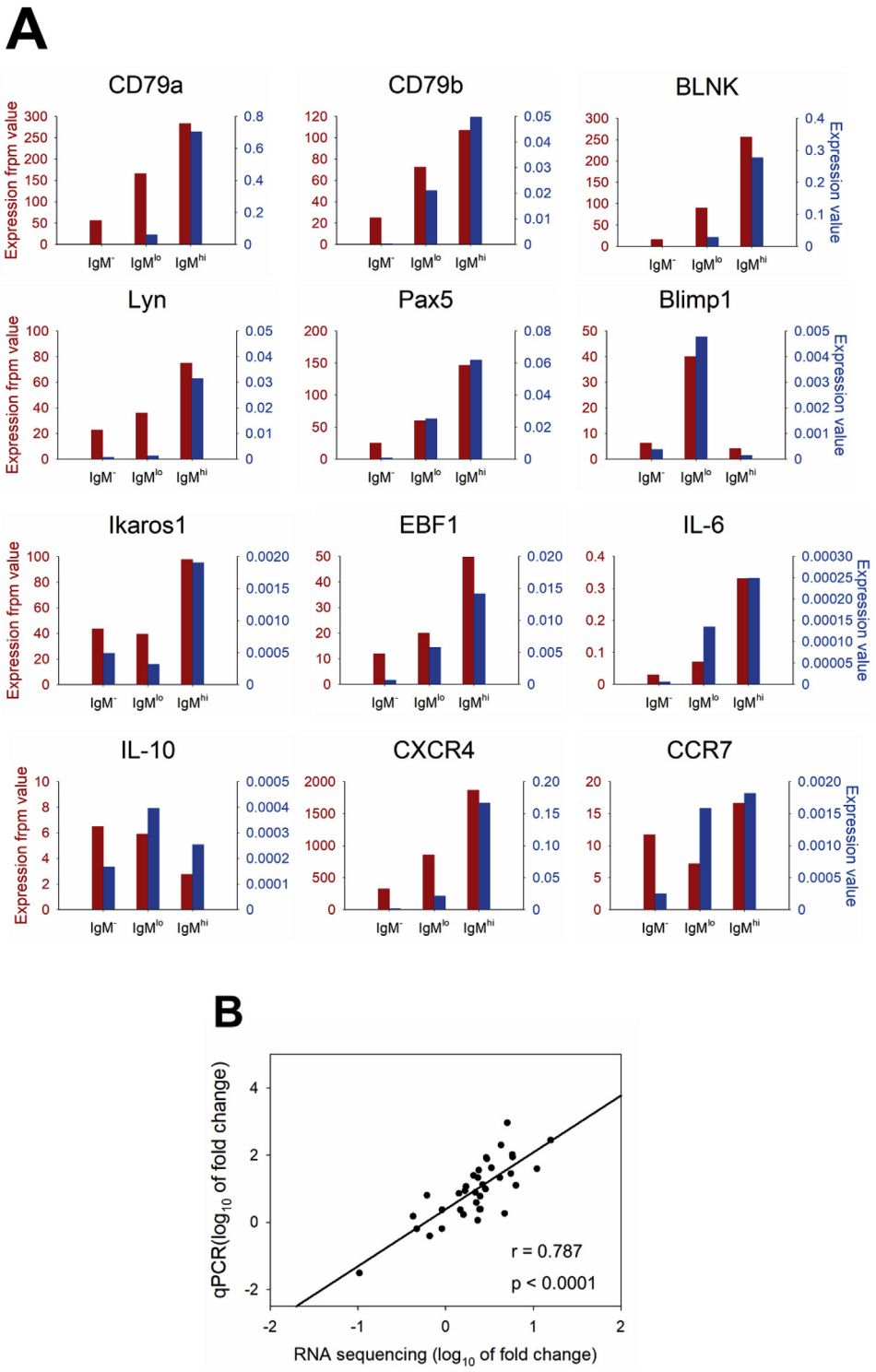

Fig. 6. Expression of the selected twelve genes inferred by RNA seq and RT-qPCR (A), and the correlation between RT-qPCR and RNA seq for the twelve selected genes (B). The relative transcript expression of three samples was shown.

encoding putative TFs were identified and categorized into 45 different TF-families (Table S14). The zf-C2H2 TF protein family was the most abundant (62 TFs, 24.22%), follow by the bHLH (26 TFs, 10.16%), TF-bZIP (24 TFs, 9.38%) and other TF families (Fig. 5A). Among these transcription factors, the B cell development related TFs, including Ikaros1, EBF1, Pax5 and Blimp1 and B-cell lymphoma 6 protein (Bcl-6) were showed in Fig. 5B.

3.8. RNA sequencing validation by RT-qPCR

To validate the expression data obtained from RNA sequencing, twelve genes were selected to perform RT-qPCR (Fig. 6). The results showed a strong correlation between the data of RNA sequencing and RT-qPCR ($r = 0.787$, $p < 0.001$, Fig. 6B). For all examined genes, the expression count values of transcriptome data exhibited similar expression profile at all the three subsets comparing with the results of RT-qPCR (Fig. 6A). It suggested a reliable expression reaksult generated

by RNA sequencing.

4. Discussion

In teleost fish, the main lymphocytes, IgM^+ B cells were identified in many species, playing important roles in the innate and the adaptive immunity [56,57]. Transcriptome analysis provides an efficient technique to investigate the overall gene expression and obtain a better understanding of cellular activities in organisms, including growth, development, disease, and immune defense [37,58]. Here we investigated the heterogeneity in IgM^+ cells from PBL in Nile tilapia, which indicated that IgM^{lo} and IgM^{hi} B cells are different B cell subsets according to the different expression of B cell development transcription factors. The IgM^{lo} B cells are resembled as antibody-secreting cells, while IgM^{hi} B cells resembled as naïve B cells as the report in rainbow trout [20]. The transcriptomic analysis presented here would provide insights into understanding of the heterogeneity in IgM^+ cells in teleost, and suggest that the IgM^+ cells are involved in the innate immunity.

Peripheral blood from teleost fish includes five main leucocyte types: lymphocytes, thrombocytes, monocytes, neutrophils and eosinophils [59]. Lymphocytes include natural killer cells (function in cell-mediated, cytotoxic innate immunity), T cells (for cell-mediated, cytotoxic adaptive immunity) and B cells (for humoral, antibody-driven adaptive immunity) [60]. IgM^+ B cells is the majority B cells in teleost fish and resembled as innate B cell subsets present in mammals [56]. The transcription level of mIgM in the sorted IgM^+ lymphocytes proved that the heterogeneity existed in the IgM^+ B cells preliminarily (Fig. 1B). Comparative transcriptome profiling data for different IgM^+ lymphocytes showed that there were amount of different expressed genes in IgM^-, IgM^{lo} and IgM^{hi} (Fig. 2).

By GO annotation, the annotated DEGs were mainly assigned to various terms of the biological process category (Fig. 3A), including cellular process and single-organism process for biological process, binding and catalytic activity for molecular function, cell, cell part, membrane and membrane part for cellular component. It has been suggested previously that immune-related proteins could be included within the cellular process, response to stimulus, signaling, immune system process categories and in binding and catalytic activity categories [61,62]. The significantly expressed DEGs in biological process were immune system process in IgM^- vs IgM^{hi} and IgM^- vs IgM^{lo} (Fig. 3B, Tables S2 and S3). GO distribution implied that different IgM^+ cells were involved in innate immunity [19]. The study on the B cell subsets in teleost is limited as the lack of specific markers compared to the study in mammals. However, mIgM is a useful marker to explore B cell subsets in teleost as studied in rainbow trout [19,25,63]. In IgM^{lo} vs IgM^{hi}, the term of nucleic acid binding transcription factor activity was significantly enriched (Fig. 3B, Table S5). It was reported that different $mIgM^+$ cells were resembled as naïve B cells (IgM^{hi}) and antibody-secreting B cells (IgM^{lo}) in rainbow trout based on the expression levels of Pax5 and Blimp1 [20]. Analyzed the important TFs related to B cell development helped to explore the heterogeneity between IgM^{lo} and IgM^{hi} cells (Fig. 5, Table S14). Ikaros1, EBF1, Bcl-6, Pax5 and Blimp1 were coincided with the B cell development related TFs in rainbow trout and fugu as well as in mammal B cell development [28,64,65]. What's more, we had proved that Pax5 and Blimp1 gets involved in B cell activation and maturation in Nile tilapia [66,67]. The result in this study indicated IgM^{lo} cells were higher differentiated B cells than IgM^{hi} cells as in rainbow trout [20].

KEGG databases for further analysis of functional annotations revealed that the DEGs in IgM^- vs IgM^{hi}, IgM^- vs IgM^{lo} and IgM^{lo} vs IgM^{hi} comparison were mainly annotated with immune system-related pathways, signaling molecules and interaction and signal transduction (Fig. 4, Table 2, S5-S7 and S15). In Intestinal immune network for IgT production pathway, there included a series of tumor necrosis factor receptor superfamily member with a significantly different expression in IgM^- cells compared to IgM^+ (IgM^{lo} and IgM^{hi}) cells, but little difference expression in IgM^{lo} vs IgM^{hi} cells. In addition, DEGs include cytokines, including IL-10 and IL-6, which were required to immune regulation and terminal differentiation process of the B cells (Table S16) [42,68]. TLRs are key molecules of the innate immune system alerting the presence of microbial pathogens through recognition of pathogen-associated molecular patterns (PAMPs), as well as a major family of pattern recognition receptors (PRRs) [69–71]. Including TLRs, NOD-like receptors (NLRs) and RIG-I-like receptors (RLRs) are the PRRs [72,73], which are intracellular cytosolic sensors [70,71,74]. There had reported a lot of different TLRs, including TLR-1, -2, -3, -5, -7, -8a, −9 and −22 in teleost fish [19]. In the present study, TLR-2, -3, -7, -8 and -9 were up-regulated DEGs in IgM^- vs IgM^{hi} (Table S16), which indicated that IgM^{hi} B cells may get involved in innate immunity as reported in rainbow trout [19]. NLRs family can sense signals and form inflammasomes when inflammatory response happens [74]. The down-regulated or up-regulated DEGs in IgM^- vs IgM^+ groups (Table S16), demonstrating that IgM^+ B cells may take parts in NOD-like receptor signaling pathway. RLRs family of PRRs is a group of cytosolic RNA helicase proteins that can identify viral RNA as non-self via binding to PAMP motifs within RNA ligands [75]. The DEGs existed in IgM^- vs IgM^+ indicated that IgM^+ lymphocytes were involved in RIG-I-like receptor signaling pathway. In signal molecules and interaction, we focused on the immune-related DEGs in cytokine-cytokine receptor interaction and discovered a lot of chemokine family and receptors. Chemokines control migration and positioning of immune cells in exposed tissues by binding to the corresponding receptors on the surface of cell target. They are also critical elements in the innate immune system and cross-talk between innate and adaptive players [76]. The different expressed chemokines in the three groups indicated that IgM^+ cells may play an important role in the triggering of innate responses after pathogen sensing as reported in rainbow trout [19]. In Jak-STAT signaling pathway, we could observe that the DEGs gene expression were down-regulated in IgM^- vs IgM^+ cells (Table S16). These data indicated that IgM^+ B cells participate in Jak-STAT signaling pathway.

In summary, our transcriptomic data suggest that IgM^+ B cells get involved in innate immunity, and IgM^{lo} and IgM^{hi} B cells are different B cell subsets. The immune-related DEGs enriched in pattern recognition receptors (TLRs, NLRs and RLRs) and cytokines. The valuable information about the heterogeneity in IgM^+ cells would provide another perspective to identify teleost B cell subsets and illustrate the role of IgM^+ cells in innate immunity.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.08.023.

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