Fish and Shellfish Immunology 84 (2019) 979–986

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

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

Full length article

Influence of CD4-1$^+$, CD4-2$^+$ and CD8$^+$ T lymphocytes subpopulations on the immune response of B lymphocytes in flounder (*Paralichthys olivaceus*) immunized with thymus-dependent or thymus-independent antigen

Jing Xing[a,b], Keke Luo[a], Yue'e Xiao[a], Xiaoqian Tang[a], Wenbin Zhan[a,b,*]

[a] Laboratory of Pathology and Immunology of Aquatic Animals, KLMME, Ocean University of China, Qingdao, 266003, PR China
[b] Laboratory for Marine Fisheries Science and Food Production Processes, Qingdao National Laboratory for Marine Science and Technology, No. 1 Wenhai Road, Aoshanwei Town, Qingdao, China

ARTICLE INFO

Keywords:
Paralichthys olivaceus
Keyhole limpet hemocyanin
Lipopolysaccharide
T lymphocytes subpopulations
B lymphocytes

ABSTRACT

In order to elucidate the influence of T lymphocytes subpopulations on B lymphocytes immune response, in this paper, CD4-1$^+$, CD4-2$^+$, CD8$^+$ T lymphocytes and B lymphocytes responses to thymus-independent (TI) or thymus-dependent (TD) antigen plus immunosuppressant were investigated in flounder (*Paralichthys olivaceus*). The results showed that in LPS-immunized group, the percentages of CD4-1$^+$, CD4-2$^+$, CD8β$^+$ T (PCD4-1$^+$ T, PCD4-2$^+$ T and PCD8β$^+$ T) lymphocytes in peripheral blood leucocytes (PBLs) had no significant variations, the percentages of IgM$^+$ B (PIgM$^+$ B) lymphocytes and LPS-specific antibodies (LA) significantly increased and peaked at 3rd or 4th week post-injection; CsA had no inhibition on both T/B lymphocytes and LA; RaPa only suppressed the PIgM$^+$ B lymphocytes and LA, and the inhibition maximum (Imax) were about 35% and 20%, respectively. In KLH-immunized group, the PCD4-1$^+$, PCD4-2$^+$ and PCD8β$^+$ T lymphocytes significantly increased and peaked at 3rd or 5th day, successively the PIgM$^+$ B lymphocytes and KLH-specific antibodies (KA) significantly increased to the peak at 5th week; the PCD4-1$^+$, PCD4-2$^+$ T and PIgM$^+$ B lymphocytes and LA were inhibited significantly by both CsA and RaPa, and the Imax on them were 13%–33%, 11%–25%, 19%–34%, 22%–26%, respectively, while the PCD8β$^+$ T lymphocytes showed no significant suppression. The results indicated that the suppression of PIgM$^+$ B lymphocytes in KLH + CsA group was not directly derived from CsA, but due to the suppression of T lymphocytes, especially CD4$^+$ T lymphocytes subpopulations. The results showed for the first time that, similar to higher vertebrates, T lymphocytes didn't respond to TI antigen, moreover, T lymphocyte subpopulations had a regulation on the immune response of B lymphocyte for TD antigen in flounder.

1. Introduction

The thymus-dependent (TD) and thymus-independent (TI) antigens are defined as whether T cells are involved in the immune response process. Keyhole Limpet Hemocyanin (KLH) is a typical TD antigen activating the cellular and humoral immune responses in mammals [1,2]. Recent studies found that dinitrophenol (DNP)-KLH or fluorescein isothiocyanate (FITC)-KLH as a protein-hapten antigen can induce serum or mucosal antibodies production in teleost [3,4]. Lipopolysaccharide (LPS) is a classical B-cell mitogen and TI antigen inducing the humoral response with increasing serum antibodies levels [5,6]. LPS increased significantly the expression of immune-related genes and induced antibodies production in fish [7,8]. Additionally, it can enhance proliferation of sIg$^+$ lymphocytes and induce apoptosis in fish lymphocytes by stimulation with LPS [9,10]. Cyclosporine A (CsA), an immunosuppressant, specifically inhibits mammalian T cells by preventing activation of transcription factors (termed nuclear factor of activated T cells (NFAT)) involved in cytokine genes expression [11], and Rapamycin (RaPa) is a crucial regulator of cell metabolism and proliferation by forming the FK506-binding protein of 12 kDa (FKBP12, a type of immunophilin)-rapamycin complex to inhibit the activation of mammalian target of RaPa (mTOR) [12]. Furthermore, CsA and RaPa have proved to suppress the T/B lymphocytes and immune-related genes associated with lymphocytes in fish [13,14].

T lymphocytes are subdivided into helper T lymphocytes (Th) and cytotoxic T lymphocytes (CTLs), defined by the expression of CD4 and CD8 glycoprotein, respectively. Th (CD4$^+$ T lymphocytes) recognizes antigens by antigen-presenting cells (APC) with MHC II and facilitates

* Corresponding author. Fisheries College, Ocean University of China, Qingdao, 266003, PR China.
E-mail address: wbzhan@ouc.edu.cn (W. Zhan).

https://doi.org/10.1016/j.fsi.2018.11.004
Received 28 June 2018; Received in revised form 31 October 2018; Accepted 1 November 2018
Available online 02 November 2018
1050-4648/

B-cells maturation and antibodies production. CTLs ($CD8^+$ T lymphocytes) recognize antigens by APC with MHC I and play a pivotal role in immunosurveillance against virus-infected or transformed cells. Similar to higher vertebrates, in fish adaptive immunity, T and B lymphocytes play a crucial role with participating in the cellular and humoral immunity, respectively and T lymphocytes influenced the immune response of B lymphocytes [13,15,16]. In recent years, two distinct CD4 molecules, CD4 (CD4-1) and CD4REL (CD4L or CD4-2), and CD8 (α and β chains) surface molecules on teleost T lymphocytes have been identified. The CD4 or CD8 molecules showed high homology and varied slightly among fish species. The characterization of CD4, CD8α and CD8β molecules was reported in Atlantic halibut (*Hippoglossus hippoglossus*) [17,18], $CD4\text{-}1^+$, $CD4\text{-}2^+$, CD4 double-positive and $CD8\alpha^+$ cells were identified in rainbow trout (*Oncorhynchus mykiss*) [19,20], CD4L-1, CD4L-2, CD8α and CD8β genes were expressed in catfish (*Ictalurus punctatus*) [21,22], three CD4 homologues (a CD4-1 molecule and two CD4-2 molecules) were found in zebrafish (*Danio rerio*) [23], and CD4-1, CD4-2, CD8α and CD8β genes were cloned and three $CD4^+$ ($CD4\text{-}1^+$ $CD4\text{-}2^+$, $CD4\text{-}1^+$ $CD4\text{-}2^-$, $CD4\text{-}1^-$ $CD4\text{-}2^+$) and $CD8\beta^+$ lymphocytes detected in flounder (*Paralichthys olivaceus*) [24,25]. Additionally, it was demonstrated that $CD4^+$ and $CD8\alpha^+$ lymphocytes presented in the ginbuna crucian carp (*Carassius auratus langsdorfii*) and $CD4^+$ cells play a major role in a secondary humoral immune response [26–28]. Furthermore, studies showed that teleost $CD4^+$ cells expressed Th-specific cytokines and transcription factors, suggesting that these $CD4^+$ cells possess functions similar to those of mammalian Th-cells [29,30].

Monoclonal antibodies against flounder IgM and polyclonal antibodies against flounder CD3, CD4-1, CD4-2 and CD8β have been prepared previously in our laboratory [25,31,32]. Additionally, T lymphocytes have proven to influence the immune response of B lymphocytes using the polyclonal antibodies against flounder CD3 [13]. In this paper, the variations of $CD4\text{-}1^+$, $CD4\text{-}2^+$, $CD8\beta^+$ T lymphocytes, IgM^+ B lymphocytes and serum specific antibodies were investigated after flounder were immunized with KLH or LPS mixed with Cyclosporine A (CsA) or Rapamycin (RaPa), respectively. The aim is to elucidate whether T lymphocytes subpopulations have an influence on the immune response of B lymphocytes in flounder.

2. Materials and methods

2.1. Experimental animals

Healthy flounder (*Paralichthys olivaceus*, body length: 13 ± 2 cm) were purchased from a marine farm (Rizhao, Shandong, China). Then they were maintained in tanks with aerated running seawater at 21 ± 1 °C and fed with commercial dry food pellets daily. After two weeks, they were used for the immunization experiments. In this study, the treatment of fish was strictly consistent with the procedures in the Guide for the Use of Experimental Animals of the Ocean University of China, and all possible effort was dedicated to minimizing suffering.

2.2. Reagents and antibodies

Keyhole Limpet Hemocyanin (KLH, cat No. H7017), Lipopolysaccharide (LPS, cat No. L2880), Cyclosporine A (CsA, cat No. C3662) and Rapamycin (RaPa, cat No. V900930) were purchased from Sigma, USA, and stored at 4 °C or −20 °C according to the instructions.

Mouse anti-flounder IgM monoclonal antibodies (FIgM-Mab), rabbit anti-flounder CD4-1 and CD4-2 polyclonal antibodies (FCD4-1-Pab, FCD4-2-Pab) and mouse anti-flounder CD8β polyclonal antibodies (FCD8β-Pab) were produced previously in our laboratory [25,31]. In this study, FIgM-Mab diluted into 1:1000 with PBS were used in Flow cytometry (FCM) and ELISA, and FCD4-1-Pab, FCD4-2-Pab and FCD8β-Pab were diluted into 1:1000, 1:1500 and 1:100 with PBS, respectively, and used in FCM. The working concentrations of antibodies were determined by pre-experiments using their gradient dilutions.

2.3. Fish immunization experiments

The administration doses were determined to induce effect immune responses in flounder, and KLH (200 μg $fish^{-1}$), LPS (200 μg $fish^{-1}$), CsA (100 μg $fish^{-1}$) and RaPa (20 μg $fish^{-1}$) were diluted in PBS [13]. Fish were randomly assigned to seven groups (180 fish $group^{-1}$), and then intraperitoneally injected with a mixture of KLH and CsA (KLH + CsA group), a mixture of KLH and RaPa (KLH + RaPa group), single KLH (KLH group), a mixture of LPS and CsA (LPS + CsA group), a mixture of LPS and RaPa (LPS + RaPa group), single LPS (LPS group), respectively. As the negative control, fish were injected with PBS of the same volume (PBS group).

2.4. Preparations of leukocytes and serum

Twelve individuals in each group were randomly sampled at 1^{st}, 3^{rd}, 5^{th}, 7^{th}, 14^{th}, 21^{st}, 28^{th}, 35^{th} and 42^{th} day, respectively. The leukocytes in peripheral blood (PBLs), spleen (SPLs), and head kidney (HKLs) were isolated according to the method as described previously [31]. Briefly, after anaesthetized with MS-222, the peripheral blood was collected from the caudal vein with 65% RPMI-1640 containing 20 IU mL-1 heparin, 0.1% w/v NaN_3 and 1% w/v BSA. The head kidney and spleen were removed and cell suspensions were prepared by squeezing the tissues with 65% RPMI-1640 described as above on a stainless mesh. Then the peripheral blood and cell suspensions were centrifuged and the supernatants were laid over a 1.020–1.070 g/cm^3 discontinuous Percoll density gradient (Sigma). After centrifuged at 840 × *g* for 30 min, the cell layers from the Percoll interface were collected and washed three times with PBS containing 5% (v/v) new-born calf serum. Finally, the leukocytes from every four individuals were pooled and adjusted to 1.0×10^6 cells mL^{-1} with PBS, respectively, and then used in FCM.

For serum preparation, blood was collected from nine individuals in each group at 1^{st}, 2^{nd}, 3^{rd}, 4^{th}, 5^{th} and 6^{th} week post-injection, respectively, and then serum were isolated according to the method as previously described [33]. Finally, the serum from every three individuals were pooled, and used in ELISA.

2.5. Flow cytometry

The PBLs, SPLs and HKLs were incubated with FIgM-Mab, FCD4-1-Pab, FCD4-2-Pab and FCD8β-Pab as the primary antibody at 37 °C for 1 h, respectively and either FITC-conjugated goat-anti-mouse IgG (1:256, Sigma, USA) or Alexa Fluor® 647-conjugated goat-anti-rabbit IgG (1:1000, Thermo Fisher Scientific, USA) as the secondary antibody in the dark at 37 °C for 45 min. After each incubation, they were washed three times with PBS containing 5% (v/v) Newborn Calf Serum. Finally, the cells suspensions were analyzed by Accuri C6 flow cytometer (BD, USA). The myeloma culture supernatant instead of FIgM-Mab and non-immunized rabbit or mouse serum instead of rabbit or mouse polyclonal antibodies were used as negative control. The experiments were repeated thrice.

2.6. Detection of serum specific antibodies by ELISA

ELISA was carried out according to a previous method [13]. In brief, KLH or LPS (5 μg mL^{-1} diluted in ddH_2O) were coated on microplates (Costar, 96-well) overnight at 4 °C. After washed three times with PBST (PBS containing 0.05% Tween-20), they were blocked with 3% BSA at 37 °C for 1 h. Then they were incubated with serum (1:100 diluted in PBS) as primary antibody at 37 °C for 1 h, FIgM-Mab (1:1000 diluted in PBS) as secondary antibody at 37 °C for 1 h, and goat-anti-mouse Ig-alkaline phosphatase conjugate (diluted 1:5000 in PBS, Southern Biotech) as third antibody at 37 °C for 1 h. After each incubation, they were

washed three times as above, and then 0.1% (w/v) p-nitrophenyl phosphate (pNPP, Sigma, USA) was added and incubated for 30 min at room temperature in the dark. Finally, the absorbance was measured at 405 nm by an automatic ELISA reader (Molecular Devices). The experiments were repeated thrice.

2.7. Statistics

All the data were analyzed using Statistical Product and Service Solution (SPSS) 20.0 software (IBM, Armonk, NY, USA) with a one-way analysis of variance (ANOVA) and graphics rendering was done with OriginPro 8.0. The results were shown as the mean ± SEM, and differences were considered significant at $p < 0.05$. Additionally, the inhibition rate was calculated according to the following formula: Inhibition rate = (values in KLH/LPS groups – that in KLH/LPS + CsA/RaPa groups)/values in KLH/LPS groups × 100%. The inhibition maximum (Imax) was the maximum inhibition rates in KLH/LPS + CsA/RaPa groups.

3. Results

3.1. The analysis of gating strategy for flow cytometry

The leukocytes from the peripheral blood, spleen and head kidney after labeled by FIgM-Mab, FCD4-1-Pab, FCD4-2-Pab or FCD8β-Pab were analyzed by flow cytometry. The cell granularity and cell size were indicated by Side-scatter (SSC) and Forward-scatter (FSC) parameters, respectively, and the major cells population containing 10,000 cells was gated to analyze the positive percentage of lymphocytes. FITC-labeled cells and Alexa Fluor® 647-labeled cells were determined with Fluorescent light (FL)-1 and FL-4, respectively. The gated lymphocytes in SSC/FSC dot plots and fluorescence histograms for the maximum of CD4-1$^+$, CD4-2$^+$, CD8β$^+$ T and IgM$^+$ B lymphocytes were shown in Fig. 1.

3.2. Variations of CD4-1$^+$, CD4-2$^+$ and CD8β$^+$ T lymphocytes after LPS immunization

After LPS immunization, variations of the PCD4-1$^+$, PCD4-2$^+$ and PCD8β$^+$ T lymphocytes in PBLs were consistent and shown in Fig. 2. The PCD4-1$^+$, PCD4-2$^+$ and PCD8β$^+$ T lymphocytes showed no significant increase and remained a relatively stable level, similar to that in control group throughout the experiment ($p < 0.05$). In addition, in LPS + CsA and LPS + RaPa groups, CsA or RaPa had no significant suppression on the PCD4-1$^+$, PCD4-2$^+$ and PCD8β$^+$ T lymphocytes when compared to that in LPS group ($p < 0.05$).

3.3. Variations of IgM$^+$ B lymphocytes and serum specific antibodies after LPS immunization

After LPS immunization, the PIgM$^+$ B lymphocytes and LA significantly increased on the 1st week or 2nd week and reached the peak on the 3rd or 4th week, then decreased gradually until the end of the experiment ($p < 0.05$). From the 1st week to the 6th week, the PIgM$^+$ B lymphocytes in LPS + RaPa group were lower than that in both LPS + CsA and LPS groups, but there was no significant difference between LPS + CsA and LPS groups ($p < 0.05$). From the 2nd week to the 6th week, the LA in LPS + RaPa group were lower than that in LPS + CsA and LPS groups, and there was no significant difference between LPS + CsA and LPS groups ($p < 0.05$) (Fig. 3). Additionally, the peak value of PIgM$^+$ B lymphocytes and LA and the Imax of CsA and RaPa were presented in Table 1.

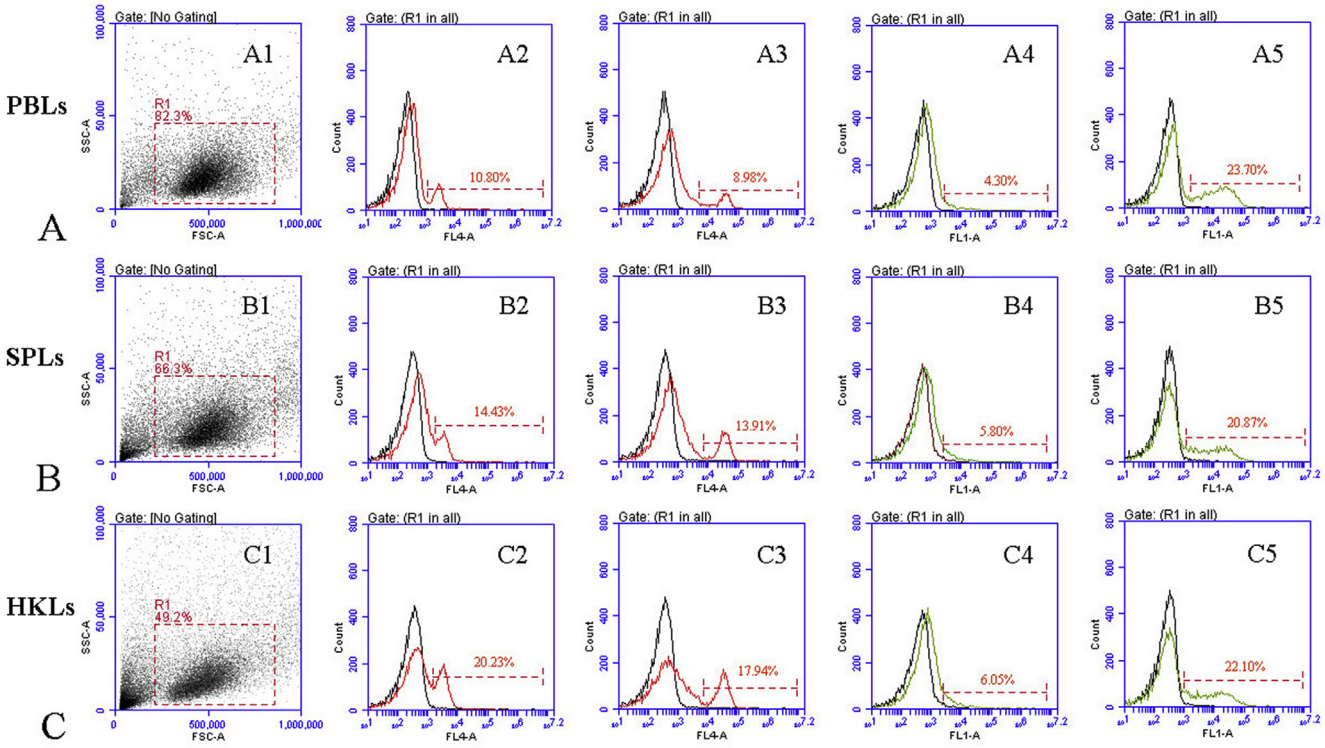

Fig. 1. Flow cytometry analysis of lymphocytes reacted with rabbit anti-flounder CD4-1 and CD4-2 polyclonal antibody, mouse anti-flounder CD8β polyclonal antibody and mouse anti-flounder IgM monoclonal antibody, respectively. A1, B1, C1: Side-scatter (SSC)/Forward-scatter (FSC) dot plots in peripheral blood leucocytes (PBLs), spleen leucocytes (SPLs) and head kidney leucocytes (HKLs), respectively; A2-A5, B2-B5, C2-C5: fluorescence histograms of the maximum of CD4-1$^+$, CD4-2$^+$, CD8β$^+$ T and IgM$^+$ B lymphocytes, respectively.

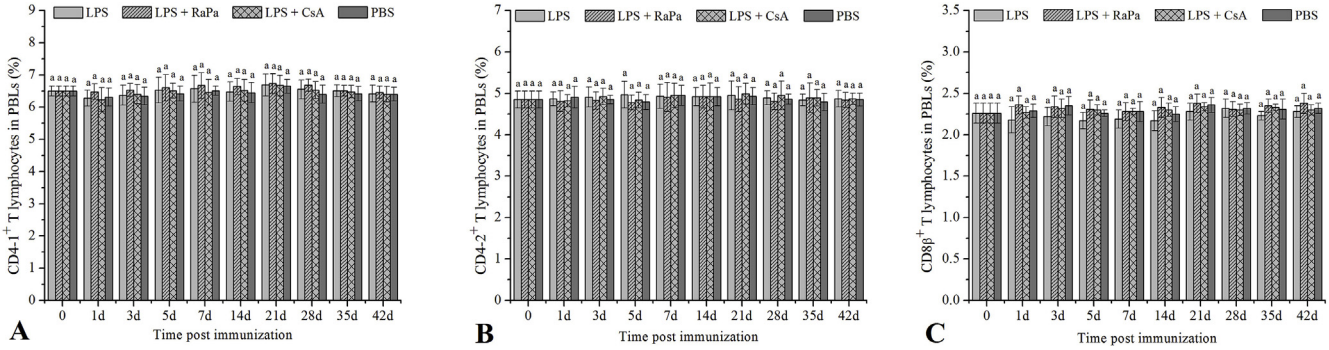

Fig. 2. Variations of CD4-1$^+$, CD4-2$^+$ and CD8β$^+$ T lymphocytes in PBLs after immunization with LPS + CsA, LPS + RaPa and LPS, respectively. The results are presented as mean ± SEM (n = 3). Different letters on the bars indicate the statistical significance at each time point ($p < 0.05$). A: CD4-1$^+$ T lymphocytes; B: CD4-2$^+$ T lymphocytes; C: CD8β$^+$ T lymphocytes.

3.4. Variations of CD4-1$^+$, CD4-2$^+$ and CD8β$^+$ T lymphocytes after KLH immunization

After KLH immunization, the PCD4-1$^+$, PCD4-2$^+$ and PCD8β$^+$ T lymphocytes in PBLs, SPLs and HKLs significantly increased at the 1st day and reached the peak on the 3rd or 5th day, then decreased gradually until the 21st or 28th day and maintained a similar level to the negative control group until the end of the experiment ($p < 0.05$). The PCD4-1$^+$ and PCD4-2$^+$ T lymphocytes in KLH + CsA and KLH + RaPa groups were significantly lower than that in KLH group at the 3rd - 7th day ($p < 0.05$). However, the PCD8β$^+$ T lymphocytes showed no significant difference among KLH + CsA, KLH + RaPa and KLH groups, ($p < 0.05$) (Fig. 4). And the peak value of PCD4-1$^+$, PCD4-2$^+$ and PCD8β$^+$ T lymphocytes and the Imax of CsA and RaPa in PBLs, SPLs and HKLs were shown in Table 2.

3.5. Variations of IgM$^+$ B lymphocytes and serum specific antibodies after KLH immunization

After KLH immunization, the PIgM$^+$ B lymphocytes and KA significantly increased on the 1st week and peaked on the 5th week, then showed reduction gradually until the end of the experiment ($p < 0.05$). From the 2nd week to the 6th week, the PIgM$^+$ B lymphocytes in both KLH + CsA and KLH + RaPa groups were significantly low compared with KLH group ($p < 0.05$). And KA in both KLH + CsA and KLH + RaPa groups was lower than that in KLH group during the 1st - 6th week (Fig. 5). The peak value of PIgM$^+$ B lymphocytes and KA and the Imax of CsA and RaPa in PBLs, SPLs and HKLs were presented in Table 2.

4. Discussions

In this study, KLH or LPS, as TD or TI antigen, were selected to induce the immune responses of T and B lymphocytes in flounder. The results showed that for KLH immunization, CD4-1$^+$, CD4-2$^+$, CD8β$^+$ T and IgM$^+$ B lymphocytes and KA significantly increased, and for LPS immunization, only IgM$^+$ B lymphocytes and LA showed a significant increase. The results were consistent with that in mammals, suggesting that KLH and LPS worked well as TD or TI antigen, respectively for this study. Similarly, in other fish species, CD4$^+$ T cells remarkably increased when CD4$^+$ T cells sorted from Zebrafish (*Danio rerio*) treated with KLH *in vivo* were co-cultured with KLH-loaded dendritic cells (DCs) [34]. In fugu (*Takifugu rubripes*), surface-Ig (sIg)$^+$ lymphocytes showed mitogenesis by stimulation with LPS, but CD8$^+$ lymphocytes/thrombocytes didn't respond to LPS [9].

After KLH immunization, CD4-1$^+$, CD4-2$^+$, CD8β$^+$ T lymphocytes in PBLs, SPLs and HKLs significantly increased within 7 days. Interestingly, CD8β$^+$ T lymphocytes responded not as much as CD4-1$^+$ and CD4-2$^+$ T lymphocytes. Some recent studies on T-cell immunity have suggested that teleost CD8$^+$ cytotoxic T cells (CTLs) play a pivotal role in antiviral immunity and protecting against intracellular bacteria infections, similar to that in mammals [35–37]. Additionally, as the model antigen of TD antigen, KLH can activate mouse immune cells and enhance the activity of CD4$^+$ T cells more than CD8$^+$ T cells [38].

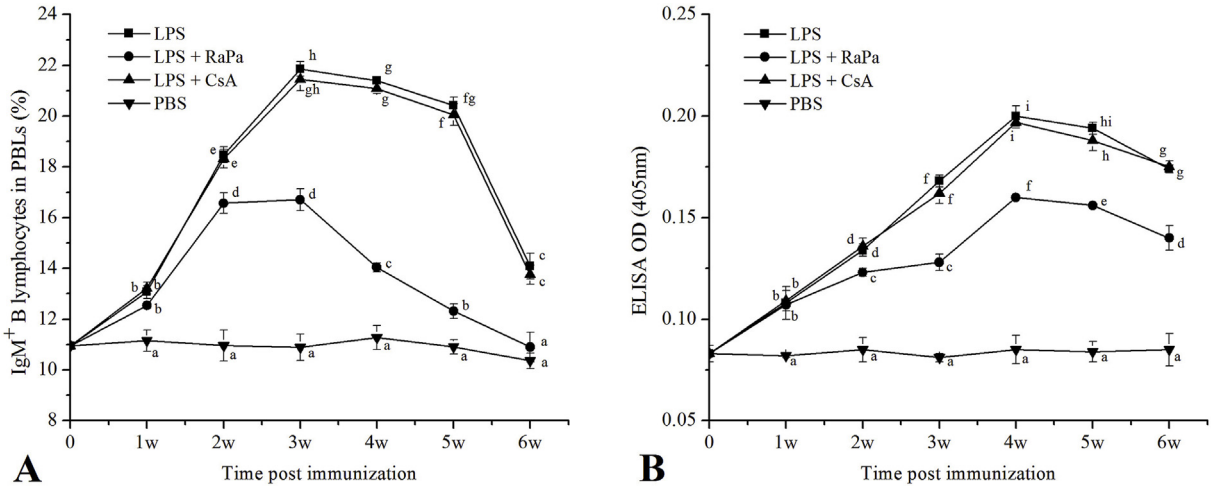

Fig. 3. Variations of IgM$^+$ B lymphocytes in PBLs and LPS-specific antibodies after immunization with LPS + CsA, LPS + RaPa and LPS, respectively. The results are presented as mean ± SEM (n = 3). Different letters on the bars indicate the statistical significance at each time point ($p < 0.05$). A: IgM$^+$ B lymphocytes; B: LPS-specific antibodies.

Table 1
The peak value of CD4-1$^+$, CD4-2$^+$, CD8β$^+$ T and IgM$^+$ B lymphocytes and LPS-specific antibodies in three LPS-immunized groups and the inhibition maximum (Imax) of CsA and RaPa.

		LPS + CsA group		LPS + RaPa group		LPS group
		peak (mean ± SEM)	Imax (mean ± SEM)	peak (mean ± SEM)	Imax (mean ± SEM)	peak (mean ± SEM)
CD4-1$^+$ T lymphocytes	PBLs	6.67% ± 0.32%	–	6.73% ± 0.31%	–	6.69% ± 0.34%
CD4-2$^+$ T lymphocytes	PBLs	4.99% ± 0.25%	–	4.92% ± 0.27%	–	4.97% ± 0.32%
CD8β$^+$ T lymphocytes	PBLs	2.32% ± 0.11%	–	2.38% ± 0.12%	–	2.35% ± 0.09%
IgM$^+$ B lymphocytes	PBLs	21.86% ± 0.29%	–	16.71% ± 0.43%*	35.21% ± 0.25%*	21.46% ± 0.47%
LPS-specific antibodies		0.194 ± 0.003	–	0.160 ± 0.001*	20.21% ± 2.23%*	0.197 ± 0.001

*represented the statistical significant difference when compared to that in LPS group (ANOVA, $p < 0.05$).
- represented that there was no Imax shown.

FIgM-Mab, FCD4-1-Pab, FCD4-2-Pab and FCD8β-Pab were crucial probes in this study, and they made it possible to analyze the IgM$^+$ B cells and CD4-1$^+$, CD4-2$^+$, CD8β$^+$ T cell subpopulations in flounder, respectively. Our previous work has verified the specificity of the antibodies by Western blotting, FCM and immunofluorescence assay (IFA) [25,31]. For these polyclonal antibodies against CD4-1, CD4-2, CD8β, the pre-experiments for working concentration setting were performed. The final dilution concentrations used in this study were determined to ensure both the positive results and the sufficient antibodies for positive cells in FCM. Also the positive cells had slight variation compared with our previous study, it was related to fish health status, developmental stage, size, water temperature etc. [19,20,25,37].

An important result from LPS-immunized group is that CD4-1$^+$, CD4-2$^+$,CD8β$^+$ T lymphocytes had no response to LPS, and the IgM$^+$ B lymphocytes and LA showed no inhibition by CsA, indicating that CsA would not directly inhibit B lymphocytes in flounder. This is similar to that in mammals, and CsA might be T lymphocytes inhibitor. Additionally, in KLH-immunized groups, PBLs, SPLs and HKLs were

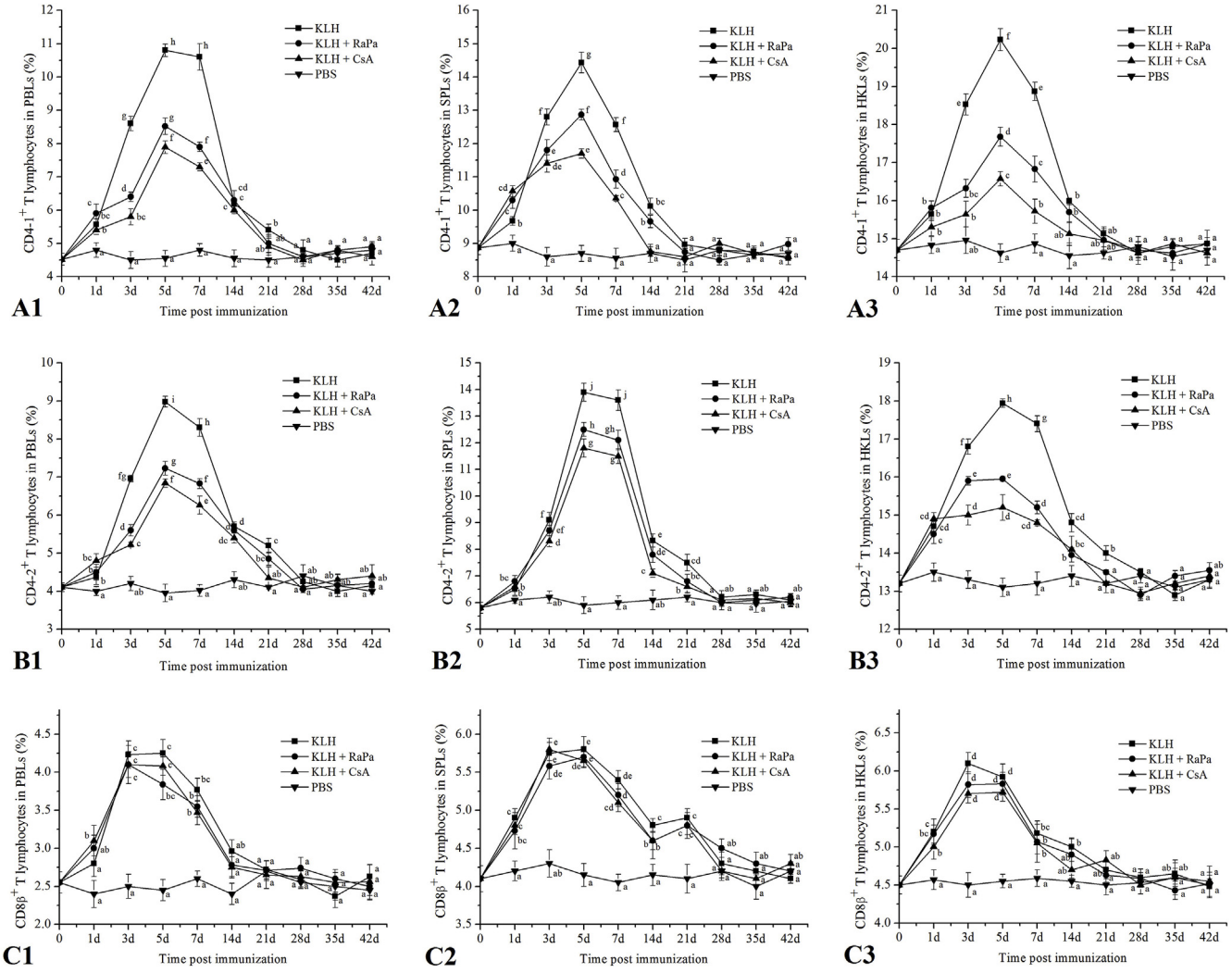

Fig. 4. Variations of CD4-1$^+$, CD4-2$^+$ and CD8β$^+$ T lymphocytes in PBLs, SPLs and HKLs after immunization with KLH + CsA, KLH + RaPa and KLH, respectively. The results are presented as mean ± SEM (n = 3). Different letters on the bars indicate the statistical significance at each time point ($p < 0.05$). A1, A2, A3: CD4-1$^+$ T lymphocytes in PBLs, SPLs and HKLs; B1, B2, B3: CD4-2$^+$ T lymphocytes in PBLs, SPLs and HKLs; C1, C2, C3: CD8β$^+$ T lymphocytes in PBLs, SPLs and HKLs.

Table 2
The peak value of CD4-1$^+$, CD4-2$^+$, CD8β$^+$ T and IgM$^+$ B lymphocytes and KLH-specific antibodies in three KLH-immunized groups and the inhibition maximum (Imax) of CsA and RaPa.

		KLH + CsA group		KLH + RaPa group		KLH group
		peak (mean ± SEM)	Imax (mean ± SEM)	peak (mean ± SEM)	Imax (mean ± SEM)	peak (mean ± SEM)
CD4-1$^+$ T lymphocytes	PBLs	7.89% ± 0.19%*	32.56% ± 1.35%*	8.52% ± 0.25%*	25.58% ± 1.15%*	10.80% ± 0.19%
	SPLs	11.70% ± 0.14%*	18.92% ± 0.68%*	12.87 ± 0.16%*	13.05% ± 0.85%*	14.43% ± 0.31%
	HKLs	16.57% ± 0.19%*	18.09% ± 0.42%*	17.68% ± 0.25%*	12.61% ± 0.56%*	20.23% ± 0.29%
CD4-2$^+$ T lymphocytes	PBLs	6.84% ± 0.11%*	25.00% ± 0.95%*	7.23% ± 0.18%*	19.54% ± 1.15%*	8.98% ± 0.14%
	SPLs	11.80% ± 0.34%*	15.44% ± 0.35%*	12.50 ± 0.26%*	11.03% ± 0.82%*	13.91% ± 0.34%
	HKLs	15.20% ± 0.34%*	15.27% ± 1.08%*	15.95% ± 0.06%*	12.64% ± 0.79%*	17.94% ± 0.11%
CD8β$^+$ T lymphocytes	PBLs	4.05% ± 0.05%	–	4.10% ± 0.08%	–	4.30% ± 0.12%
	SPLs	5.90% ± 0.08%	–	5.70% ± 0.14%	–	5.80% ± 0.23%
	HKLs	5.75% ± 0.08%	–	5.85% ± 0.07%	–	6.05% ± 0.09%
IgM$^+$ B lymphocytes	PBLs	15.80% ± 0.15%*	33.62% ± 1.47%*	16.65% ± 0.35%*	29.75% ± 1.46%*	23.70% ± 0.46%
	SPLs	15.55% ± 0.35%*	26.17% ± 0.68%*	16.32% ± 0.35%*	22.34% ± 0.54%*	20.87% ± 0.36%
	HKLs	16.65% ± 0.38%*	24.93% ± 1.27%*	18.00% ± 0.35%*	18.55% ± 0.96%*	22.10% ± 0.46%
KLH-specific antibodies in serum		0.206 ± 0.003*	25.86% ± 1.13%*	0.215 ± 0.001*	22.32% ± 1.54%*	0.277 ± 0.008

*represented the statistical significant difference when compared to that in KLH group (ANOVA, $p < 0.05$).
-represented that there was no Imax shown.

used, however, in LPS-immunized groups, only PBLs were sampled, this is because that there were no immune response for T lymphocytes to LPS and the KLH results also showed T/B lymphocytes had similar variation among PBLs, SPLs and HKLs. It could be deduced that the variations of T lymphocytes subpopulations and B lymphocytes in SPLs and HKLs were consistent with that in PBLs.

In the present study, KLH-stimulated CD4-1$^+$ T, CD4-2$^+$ T and IgM$^+$ B lymphocytes and KA were suppressed significantly by both CsA and RaPa, and LPS-stimulated IgM$^+$ B lymphocytes and LA were inhibited by RaPa, which were consistent with inhibition of CsA and RaPa on lymphocytes in mammals [39,40]. An early work showed that the cellular and antibodies response to KLH was inhibited by cyclosporin in

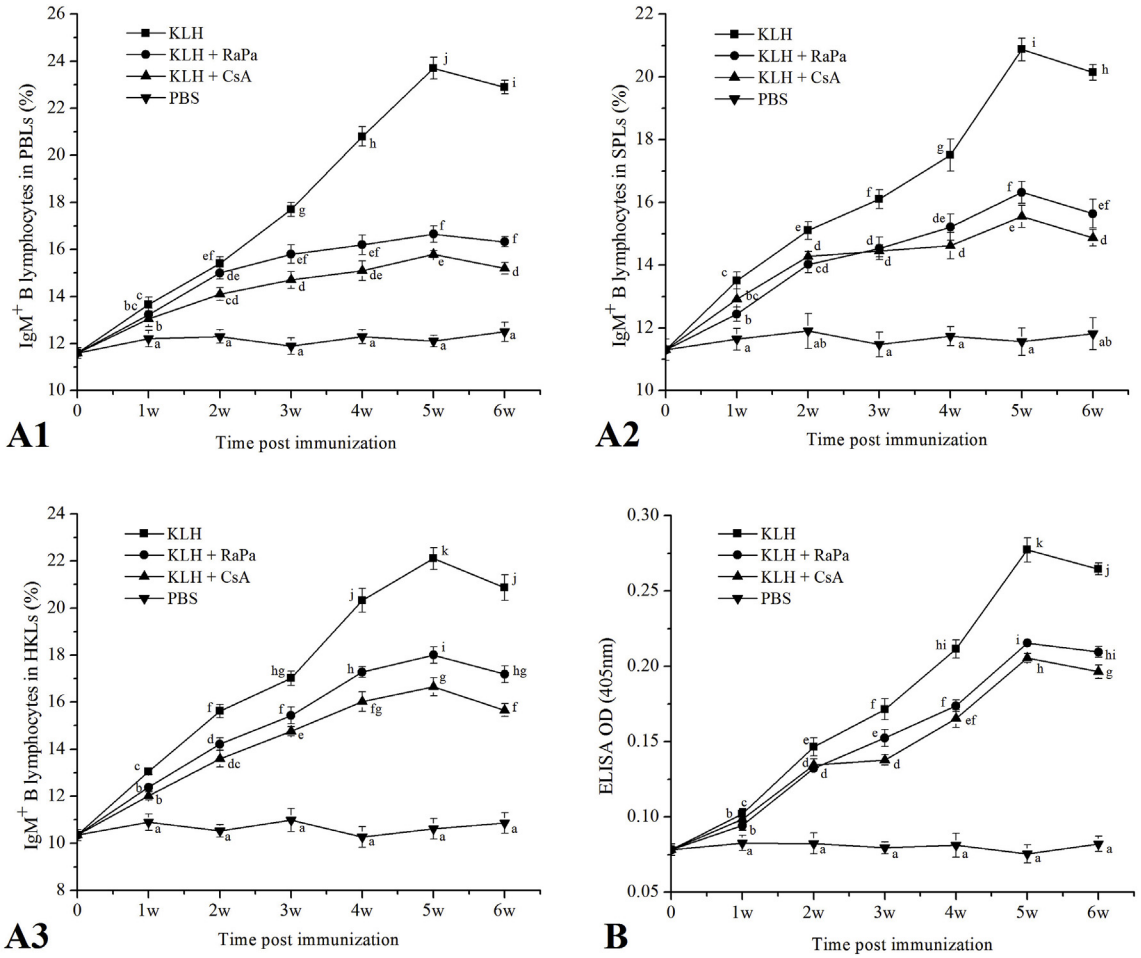

Fig. 5. Variations of IgM$^+$ B lymphocytes in PBLs, SPLs and HKLs and KLH-specific antibodies after immunization with KLH + CsA, KLH + RaPa and KLH, respectively. The results are presented as mean ± SEM (n = 3). Different letters on the bars indicate the statistical significance at each time point ($p < 0.05$). A1, A2, A3: IgM$^+$ B lymphocytes in PBLs, SPLs and HKLs; B: KLH-specific antibodies.

normal humans [41]. In KLH-immunized rats, CsA caused complete suppression of the KLH-specific IgM and IgG production, and significantly inhibited $CD3^+CD4^+$, but not $CD3^+CD8^+$ in spleen cells [42]. The proliferation of T lymphocytes after PHA stimulation was specifically inhibited by CsA, but the proliferation of B lymphocytes after LPS stimulation was not inhibited in channel catfish (*Ictalurus punctatus*), which was similar to that in this study [14]. Furthermore, it was demonstrated from the results that the inhibition of B lymphocytes and KA in KLH + CsA group were not directly derived from CsA, but due to the suppression of T lymphocytes, especially $CD4^+$ T lymphocytes subpopulations. CsA or RaPa inhibited the CD4-1^+ and CD4-2^+ T lymphocytes, then B lymphocytes immune response was suppressed, decreasing antibodies production. Additionally, our pre-experiments have found that the responses of T/B lymphocytes and immune-related genes associated with lymphocytes in PBS + CsA and PBS + RaPa groups were similar to that of PBS group, and they were no significant variations among the three groups. Therefore, in this study, negative control was used with PBS instead of PBS + CsA and PBS + RaPa.

Meantime, the study found that the immune responses of B lymphocytes and KA which peaked at 5^{th} week lagged, compared to CD4-1^+ and CD4-2^+ T lymphocytes peaked at 5^{th} day in KLH-immunized groups. A recent study also showed that CD4-1^+ and CD4-2^+ T subsets increased significantly before the response of B lymphocytes and antibodies production when stimulated with virus or inactivated-virus in flounder [37]. Based on the results here, it was further indicated that $CD4^+$ T lymphocytes had a regulation on the immune response of B lymphocytes, stimulating antibodies production in flounder [43,44]. Similarly, in clonal ginbuna crucian carp (*Carassius auratus langsdorfii*), it was demonstrated that $CD4^+$ cells help to induce a secondary antibody response [28]. Another study also demonstrated that Th-like cells clone expressing CD4-1 from common carp (*Cyprinus carpio*) shared some features with mammalian Th2 cells, suggesting that $CD4^+$ cells play an important role in humoral immunity [30]. Further study is needed to reveal the specific mechanism about the interaction of T lymphocytes subpopulations and B lymphocytes by using T lymphocytes subpopulations lineage or costimulatory molecules expressed on T and B cells surface in teleost [16,30].

Our previous study through qPCR analysis have proven that KLH could induce the upregulation of CD4-1, CD4-2, CD8-α, CD8-β, MHC I, MHC II, IgM, CD40 and IFN-γ genes in spleen and head kidney tissues, and both CsA and RaPa could inhibit their expression [13]. Moreover, RT-PCR analysis of immunomagnetic bead sorted cells with the CD4-1, CD4-2 and CD8β antibodies was performed to confirm the expression profiles of CD3ε, TCR-α, TCR-β, CD4-1, CD4-2, CD8-α, CD8-β, IgM and IgT genes in our previous work [25]. Variations of lymphocytes subpopulation related genes in sorted cells may be more supportable, however, it will be impracticable that large amounts of individuals sampled from seven groups and ten time points in this study, and it is recommended that expression of lymphocytes subpopulation related genes in sorted T cells subsets at a certain time point is detected in future research.

In conclusion, the study indicated that KLH can activate the response of $CD4^+$, $CD8^+$ T lymphocytes subpopulations and B lymphocytes, but LPS only induced B lymphocytes and antibodies production in flounder. Both CsA and RaPa could inhibit the immune response of lymphocytes, but CsA specifically suppressed T lymphocytes and then B lymphocytes immune response was suppressed. Additionally, $CD4^+$ T lymphocytes subpopulation showed a regulation on the immune response of B lymphocytes, stimulating antibodies production in flounder, which provide a good reference for the functions of T lymphocytes subpopulations and B lymphocytes in teleost.

Acknowledgements

This study was supported by the Fundamental Research Funds for the Central Universities (201822015), the NSFC (31730101; 31672684; 31672685), the OUC-AU joint projects, NBRPC (2012CB114406), Development Program of Shandong Province (2016GNC115001), and Taishan Scholar Program of Shandong Province.

References

[1] J.R. Harris, J. Markl, Keyhole limpet hemocyanin (KLH): a biomedical review, Micron 30 (1999) 597–623.
[2] V.L. Peachee, M.J. Smith, M.J. Beck, D.J. Stump, K.L. White, Characterization of the T-dependent antibody response (TDAR) to keyhole limpet hemocyanin (KLH) in the Gottingen minipig, J. Immunot. 11 (2014) 376–382.
[3] V.A. Valdenegro-Vega, P. Crosbie, B. Vincent, K.D. Cain, B.F. Nowak, Effect of immunization route on mucosal and systemic immune response in Atlantic salmon (*Salmo salar*), Vet. Immunol. Immunopathol. 151 (2013) 113–123.
[4] M.D. Lange, C.D. Webster, The effect of temperature on the mucosal IgM antibody response to DNP-KLH in channel catfish (*Ictalurus punctatus*), Fish Shellfish Immunol. 70 (2017) 493–497.
[5] A. Coutinho, G. Möller, B cell mitogenic properties of thymus-independent antigens, Nat. New Biol. 245 (1973) 12–14.
[6] L.W. Clem, N.W. Miller, J.E. Bly, Evolution of Lymphocyte Subpopulations, Their Interactions and Temperature Sensitivities, Phylogenesis of Immune Functions, (1991), pp. 191–213.
[7] J. Diao, H.B. Ye, X.Q. Yu, Y. Fan, L. Xu, et al., Adjuvant and immunostimulatory effects of LPS and β-glucan on immune response in Japanese flounder, *Paralichthys olivaceus*, Vet. Immunol. Immunopathol. 156 (2013) 167–175.
[8] Q.N. Liu, Z.Z. Xin, X.Y. Chai, S.H. Jiang, C.F. Li, et al., Characterization of immune-related genes in the yellow catfish *Pelteobagrus fulvidraco* in response to LPS challenge, Fish Shellfish Immunol. 56 (2016) 248–254.
[9] K. Araki, K. Akatsu, H. Suetake, K. Kikuchi, Y. Suzuki, Characterization of CD8(+) leukocytes in fugu (*Takifugu rubripes*) with antiserum against fugu CD8α, Dev. Comp. Immunol. 32 (2008) 850–858.
[10] L.X. Xiang, B. Peng, W.R. Dong, Z.F. Yang, J.Z. Shao, Lipopolysaccharide induces apoptosis in *Carassius auratus* lymphocytes, a possible role in pathogenesis of bacterial infection in fish, Dev. Comp. Immunol. 32 (2008) 992–1001.
[11] A. Rao, C. Luo, P.G. Hogan, Transcription factors of the NFAT family: regulation and function, Annu. Rev. Immunol. 15 (1997) 707–747.
[12] D.M. Sabatini, H.E. Bromage, M. Liu, P. Tempst, S.H. Snyder, RAF T1: a mammalian protein that binds to FKBP12 in a rapamycin-dependent fashion and is homologous to yeast TORs, Cell 78 (1994) 35–43.
[13] J. Xing, Y.E. Xiao, X.Q. Tang, X.Z. Sheng, W.B. Zhan, Inhibition of Cyclosporine A or rapamycin on T lymphocyte counts and the influence on the immune responses of B lymphocytes in flounder (*Paralichthys olivaceus*), Fish Shellfish Immunol. 66 (2017) 78–85.
[14] H. Park, H. Zhou, E. Bengten, M. Wilson, V.G. Chinchar, et al., Activation of channel catfish (*Italurus punctatus*) T cells involves NFAT-like transcription factors, Dev. Comp. Immunol. 26 (2002) 775–784.
[15] R.N. Morrison, A.B. Lyons, B.F. Nowak, J.D. Hayball, Snapper (*Pagrus auratus*) leucocyte proliferation is synergistically enhanced by simultaneous stimulation with LPS and PHA, Fish Shellfish Immunol. 16 (2004) 307–319.
[16] Y.F. Gong, L.X. Xiang, J.Z. Shao, CD154-CD40 interactions are essential for thymus-dependent antibody production in zebrafish: insights into the origin of costimulatory pathway in helper T cell-regulated adaptive immunity in early vertebrates, J. Immunol. 182 (2009) 7749–7762.
[17] S. Patel, A.C. Overgård, A.H. Nerland, CD8α and CD8β in Atlantic halibut, *Hippoglossus hippoglossus*: cloning, characterization and gene expression during viral and bacterial infection, Fish Shellfish Immunol. 25 (2008) 570–580.
[18] S. Patel, A.C. Overgård, A.H. Nerland, A CD4 homologue in Atlantic halibut (*Hippoglossus hippoglossus*): molecular cloning and characterization, Fish Shellfish Immunol. 26 (2009) 377–384.
[19] F. Takizawa, J.M. Dijkstra, P. Kotterba, T. Korytář, H. Kock, et al., The expression of CD8α discriminates distinct T cell subsets in teleost fish, Dev. Comp. Immunol. 35 (2011) 752–763.
[20] F. Takizawa, S. Magadan, D. Parra, Z. Xu, T. Korytář, P. Boudinot, J.O. Sunyer, Novel teleost CD4-bearing cell populations provide insights into the evolutionary origins and primordial roles of $CD4^+$ lymphocytes and $CD4^+$ macrophages, J. Immunol. 196 (2016) 4522–4535.
[21] E.S. Edholm, J.L. Stafford, S.M. Quiniou, G. Waldbieser, N.W. Miller, E. Bengten, M. Wilson, Channel catfish, *Ictalurus punctatus*, CD4-like molecules, Dev. Comp. Immunol. 31 (2007) 172–187.
[22] S.M. Quiniou, M. Sahoo, E.S. Edholm, E. Bengten, M. Wilson, Channel catfish CD8α and CD8β co-receptors: characterization, expression and polymorphism, Fish Shellfish Immunol. 30 (2011) 894–901.
[23] S. Yoon, S. Mitra, C. Wyse, A. Alnabulsi, J. Zou, et al., First demonstration of antigen induced cytokine expression by CD4-1^+ lymphocytes in a poikilotherm: studies in zebrafish (*Danio rerio*), PloS One 10 (2015) e0126378.
[24] G. Kato, K. Goto, I. Akune, S. Aoka, H. Kondo, I. Hirono, CD4 and CD8 homologues in Japanese flounder, *Paralichthys olivaceus*: differences in the expressions and localizations of CD4-1, CD4-2, CD8α and CD8β, Dev. Comp. Immunol. 39 (2013) 293–301.
[25] J. Xing, J.J. Ma, X.Q. Tang, X.Z. Sheng, W.B. Zhan, Characterizations of CD4-1, CD4-2 and CD8β T cell subpopulations in peripheral blood leucocytes, spleen and head kidney of Japanese flounder (*Paralichthys olivaceus*), Mol. Immunol. 85 (2017) 155–165.
[26] H. Toda, Y. Shibasaki, T. Koike, M. Ohtani, F. Takizawa, et al., Alloantigen-specific

killing is mediated by CD8-positive T cells in fish, Dev. Comp. Immunol. 33 (2009) 646–652.
[27] H. Toda, Y. Saito, T. Koike, F. Takizawa, K. Araki, et al., Conservation of characteristics and functions of CD4 positive lymphocytes in a teleost fish, Dev. Comp. Immunol. 35 (2011) 650–660.
[28] T. Somamoto, M. Kondo, T. Nakanishi, M. Nakao, Helper function of CD4 (+) lymphocytes in antiviral immunity in ginbuna crucian carp, *Carassius auratus langsdorfii*, Dev. Comp. Immunol. 44 (2014) 111–115.
[29] T. Kono, H. Korenaga, Cytokine gene expression in CD4 positive cells of the Japanese Pufferfish, *Takifugu rubripes*, PloS One 8 (2013) e66364.
[30] T. Yamaguchi, F. Katakura, K. Someya, J.M. Dijkstra, T. Moritomo, T. Nakanishi, Clonal growth of carp (*Cyprinus carpio*) T cells in vitro: long-term proliferation of Th2-like cells, Fish Shellfish Immunol. 34 (2013) 433–442.
[31] Q. Li, W.B. Zhan, J. Xing, X.Z. Sheng, Production, characterisation and applicability of monoclonal antibodies to immunoglobulin of Japanese flounder (*Paralichthys olivaceus*), Fish Shellfish Immunol. 23 (2007) 982–990.
[32] X.Q. Tang, Y.H. Qin, X.Z. Sheng, J. Xing, W.B. Zhan, Characterization of $CD3^+$ T lymphocytes of Japanese flounder (*Paralichthys olivaceus*) and its response after immunization with formalin-inactivated *Edwardsiella tarda*, Fish Shellfish Immunol. 63 (2017) 220–227.
[33] Y. Du, X.Q. Tang, X.Z. Sheng, J. Xing, W.B. Zhan, Immune response of flounder (*Paralichthys olivaceus*) was associated with the concentration of inactivated *Edwardsiella tarda* and immersion time, Vet. Immunol. Immunopathol. 167 (2015) 44–50.
[34] T. Shao, L.Y. Zhu, L. Nie, W. Shi, W.R. Dong, L.X. Xiang, J.Z. Shao, Characterization of surface phenotypic molecules of teleost dendritic cells, Dev. Comp. Immunol. 49 (2015) 38–43.
[35] T. Somamoto, E.O. Koppang, U. Fischer, Antiviral functions of $CD8^+$ cytotoxic T cells in teleost fish, Dev. Comp. Immunol. 43 (2014) 197–204.
[36] M. Yamasaki, K. Araki, T. Nakanishi, C. Nakayasu, A. Yamamot, Role of $CD4^+$ and $CD8\alpha^+$ cells in protective immunity against *Edwardsiella tarda* infection of ginbuna crucian carp, *Carassius auratus langsdorfi*, Fish Shellfish Immunol. 36 (2014) 299–304.
[37] J. Xing, L. Wang, M.X. Zhen, X.Q. Tang, W.B. Zhan, Variations of T and B lymphocytes of flounder (*Paralichthys olivaceus*) after Hirame novirhabdovirus infection and immunization, Mol. Immunol. 96 (2018) 19–27.
[38] A.G. Doly, L. Ramn, A. Kelso, The $CD4^+$ T-cell response to protein immunization is independent of accompanying IFN-γ-producing $CD8^+$ T cells, Immunol. 93 (1998) 341–349.
[39] Y. Zhang, G. Cheng, Z.W. Xu, Z.L. Li, C.J. Song, et al., Down regulation of TRAIL and FasL on NK cells by Cyclosporin A in renal transplantation patients, Immunol. Lett. 152 (2013) 1–7.
[40] A. Chebel, R. Catallo, C. Mabon, E. Bachy, T. Wenner, G. Salles, C.P. Noble, M. Ffrench, Rapamycin safeguards lymphocytes from DNA damage accumulation in vivo, Eur. J. Cell Biol. 95 (2016) 331–341.
[41] P.L. Amlot, A.E. Hayes, D. Gray, E.C. Gordon-Smith, J.H. Humphrey, Human immune responses in vivo to protein (KLH) and polysaccharide (DNP-Ficoll) neoantigens: normal subjects compared with bone marrow transplant patients on cyclosporine, Clin. Exp. Immunol. 64 (1986) 125–135.
[42] D. Roman, P. Ulrich, G. Paul, M. Court, P. Vit, J. Kehren, A. Mahl, Determination of the effect of calcineurin inhibitors on the rat's immune system after KLH immunization, Toxicol. Lett. 149 (2004) 133–140.
[43] A. Jeurissen, A.D. Billiau, L. Moens, L. Shengqiao, W. Landuyt, et al., $CD4^+$ T lymphocytes expressing CD40 ligand help the IgM antibody response to soluble pneumococcal polysaccharides via an intermediate cell type, J. Immunol. 176 (2006) 529–536.
[44] C. Tafalla, E. Leal, T. Yamaguchi, U. Fischer, T cell immunity in the teleost digestive tract, Dev. Comp. Immunol. 64 (2016) 167–177.