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# Characterizations of CD4-1, CD4-2 and CD8β T cell subpopulations in peripheral blood leucocytes, spleen and head kidney of Japanese flounder (*Paralichthys olivaceus*)

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ABSTRACT

In the previous study, antibodies against CD3 molecule have been produced and were used in labeling T cells in Japanese flounder (*Paralichthys olivaceus*). In this paper, $CD4^+$ and $CD8^+$ lymphocytes subpopulations in peripheral blood leucocytes (PBL), spleen and head kidney of flounder were investigated. The flounder CD4-1, CD4-2 and CD8β recombinant proteins and their antibodies (Abs) were produced, then the cross-reactivity of the Abs to CD4-1, CD4-2 and CD8β was detected by Western blotting, respectively, and the reactions of Abs to PBL were analyzed by immunofluorescence staining (IFS) and flow cytometry (FCM). $CD4\text{-}1^+/CD3^+$, $CD4\text{-}2^+/CD3^+$, and $CD8\beta^+/CD3^+$ lymphocytes in PBL, spleen and head kidney were observed by double IFS, then their proportions were analyzed using two-color FCM, respectively. Further, CD4-1/CD8β, CD4-2/CD8β, or CD4-1/CD4-2 lymphocytes were analyzed using double-IFS and two-color FCM. Finally, $CD4\text{-}1^+$, $CD4\text{-}2^+$, and $CD8\beta^+$ lymphocytes in spleen and head kidney were observed by immunohistochemistry. The results showed that the Abs were specific for CD4-1, CD4-2 and CD8β molecules, respectively. The proportions of $CD4\text{-}1^+/CD3^+$, $CD4\text{-}2^+/CD3^+$, and $CD8\beta^+/CD3^+$ lymphocytes were $6.7 \pm 2.0\%$, $8.6 \pm 2.8\%$, $2.1 \pm 1.3\%$ in PBL; $13.6 \pm 3.6\%$, $15.6 \pm 5.2\%$, $2.8 \pm 1.4\%$ in spleen; $20.0 \pm 4.6\%$, $20.5 \pm 4.6\%$, $3.2 \pm 1.5\%$ in head kidney, respectively. Most $CD4^+$ and $CD8^+$ cell subpopulations belonged to $CD3^+$ cells; there were no cross-reactivity between $CD4^+$ and $CD8^+$ cells. $CD4\text{-}1^+/CD4\text{-}2^-$, $CD4\text{-}1^-/CD4\text{-}2^+$, and $CD4\text{-}1^+/CD4\text{-}2^+$ cells presented different proportions in PBL, spleen and head kidney, among them, $CD4\text{-}1^+/CD4\text{-}2^+$ cell is the majority of CD4T cell subpopulation.



## 1. Introduction

The CD3 molecules which are specifically expressed on T cell surface were cloned and sequenced in some teleost fishes (Park et al., 2001; Araki et al., 2005; Liu et al., 2008; Øvergård et al., 2009). In recent years, the CD4 or CD8 surface molecules were reported in rainbow trout (*Oncorhynchus mykiss*) (Dijkstra et al., 2006), sea bass (*Dicentrarchus labrax* L) (Buonocore et al., 2006), Atlantic salmon (*Salmo salar*) (Moore et al., 2005, 2009), fugu (*Takifugu rubripes*) (Suetake et al., 2007), Atlantic halibut (*Hippoglossus hippoglossus*) (Patel et al., 2008) etc. Two CD4 subsets were described: the CD4-1 molecule contains four Ig-like domains (D1-D4), a transmembrane domain, and a cytoplasmic domain. The CD4-2 molecule encodes a protein with either two or three Ig-like domains (Castro et al., 2011; Laing and Hansen, 2011). CD8 has two chains, CD8α and CD8β, which form either heterodimers (CD8αβ) or homodimers (CD8αα) (Cole and Gao, 2004). The presences of CD4 and CD8 subsets vary with fish species. CD4-1 and CD8α presented in the ginbuna crucian carp (*Carassius auratus langsdorfii*) (Toda et al., 2009, 2011a,b,c), CD8α expressed in trout tissues (Takizawa et al., 2011), CD4 in Japanese pufferfish (*Fugu rubripes*) (Kono and Korenaga, 2013), $CD4\text{-}1^+$, $CD4\text{-}2^+$ and CD4 double-positive cells in rainbow trout (*Oncorhynchus mykiss*) (Takizawa et al., 2016). In Japanese flounder (*Paralichthys olivaceus*), CD4-1, CD4-2, CD8α, and CD8β genes have been cloned and their localizations and expressions in tissues at transcriptional level were reported (Kato et al., 2013). Our previous work has produced the monoclonal antibodies against flounder IgM (FIgM-Mab, Li et al., 2007), also the recombinant protein and antibodies (Abs) of *P. olivaceus* T lymphocytes surface molecule, CD3 (CD3ε) (Zhen et al., 2017), the FIgM-Mab and CD3 Abs labeled $IgM^+$ lymphocytes and $CD3^+$ lymphocytes, respectively, and there

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was no cross-reactivity with each other. In this paper, CD4-1, CD4-2, and CD8β recombinant proteins were expressed, and their Abs were produced, respectively. Together with CD3 Abs, the distribution of CD4-1$^+$, CD4-2$^+$, and CD8β$^+$ lymphocytes in peripheral blood leucocytes, spleen and head kidney were observed by immunofluorescence staining, immunohistochemistry, and the proportions were analyzed by flow cytometry. The results will provide new knowledge to distinguish T cell subpopulations at cellular and molecular level in flounder.

## 2. Materials and methods

### 2.1. Animals and antibody

Apparently healthy flounder (*Paralichthys olivaceus*) (weight: 800–1000 g) were purchased from a fish farm in Rizhao, Shandong Province, PR China. Then fish were acclimated in aerated flowing seawater at 21 °C and fed twice a day with commercial pellets. The fish were acclimated in laboratory for 2 weeks and only healthy fish, as determined by general appearance and level of activity were used in the following experiments. New Zealand white rabbits (~3 kg) and Balb/C mice were purchased from Qingdao Animal Experimental Center (Shandong, China) and then used for antibody production. This study was carried out strictly with the procedures in the Guide for the Use of Experimental Animals of the Ocean University of China in agreement with the International Guiding Principles for Biomedical Research Involving Animals (EU 2010/63). All efforts were dedicated to minimize suffering.

Mouse anti-flounder IgM monoclonal antibodies (FIgM-Mab, 1:1000 diluted in PBS) were produced previously in our laboratory and the specificity to IgM$^+$ B cells was verified (Li et al., 2007) and in this paper, the ascite fluids were used in flow cytometry (FCM). The FIgM-Mab were also used to localize the lymphocytes population size and granularity on the forward scatter (FSC)/side scatter (SSC) plots in FCM analysis. The CD3 recombinant protein and rabbit anti-flounder CD3 Abs were preserved in our lab, CD3 Abs were proved to be specific to CD3 molecule using western blotting and Mass spectroscopy (Zhen et al., 2017). The CD3 recombinant protein was used in Western blotting (WB), CD3 Abs were diluted into 1:1000 and used in immunofluorescence staining (IFS) and FCM.

### 2.2. Preparation of leukocytes, tissue cDNAs and tissue sections

The leukocytes in peripheral blood, spleen, and head kidney were isolated according to the protocols as described in our laboratory before (Li et al., 2007). The isolated leukocytes were used in IFS, FCM, and WB. Spleen and head kidney were dissected and the total RNA was extracted by Trizol method, then first strand cDNA was synthesized using RNA reverse transcript kits (TAKARA, Japan), the cDNA samples were used for recombinant proteins production.

For tissue sections, spleen and head kidney were fixed in Bouin's solution at room temperature for 24 h. And then rinsed in 70% alcohol, embedded in paraffin, and sectioned with a thickness of 5–7 um, finally were mounted on APES (amino-propyl-tri-ethoxy-silane) coated slides (Citotest, China). The slides were dehydrated, deparaffinized and rehydrated, then used in immunohistochemistry (IHC).

### 2.3. Production of flounder CD4-1, CD4-2, CD8α and CD8β recombinant proteins and the antibodies

The cDNA sequences encoding *P. olivaceus* extracellular domains except the signal peptide of CD4-1 (GenBank accession no. AB643634), CD4-2 (GenBank accession no. AB640684), CD8α (GenBank accession no. AB082957), and CD8β (GenBank accession no. AB643633) were amplified, and the specific primers used were listed in Table 1. The PCR products were cloned into pMD19-T Vector (TAKARA, Japan), after confirmation as the right sequences, they were cloned into digested pET28a vectors, the recombinant plasmids were transformed into *Escherichia coli* BL21 Star (DE3)–competent cells (Invitrogen/Life Technologies) and induced during exponential growth with isopropylb-D-thiogalactopyranoside (cd4-1, 0.5 mmol/L; cd4-2, 1.0 mmol/L, cd8α, cd8β 0.8 mmol/L) for 4 h at 30 °C. Finally, the recombinant proteins were affinity-purified using His Trap$^{TM}$ HP Ni-Agarose (GE healthcare China, Beijing, China) according to the manufacturer's instructions. The concentrations of the purified proteins were determined by the Bradford method.

Purified recombinant proteins were used for immunization. Briefly, New Zealand white rabbits were immunized with 600 ug CD4-1 or CD4-2 recombinant protein, and the mice were immunized with 50 ug CD4-1, CD4-2 or CD8β recombinant proteins, respectively. After three boosters, the serum was collected, and then the antibodies (Abs) were purified with protein G–agarose column (Pierce/Thermo Scientific). The titers were tested by ELISA,

**Table 1**
Primers used in this study.

| Genes | Accession No. | Sequence | |
|---|---|---|---|
| CD4-1 | AB643634 | F | 5′-CCGGAATTCAGTGAGGTGGTTTATGCT-3′ (EcoR I) |
| CD4-1 | | R | 5′-CCCAAGCTTCCACACATTCAGTTTGGTTTC-3′ (Hind III) |
| CD4-2 | AB640684 | F | 5′-CCGGAATTCGCAGCCACTGTGATCCTAAC −3′ (EcoR I) |
| CD4-2 | | R | 5′-CCCAAGCTTGGGTGTTACAGCAGGTTCTT −3′ (Hind III) |
| CD8-α | AB082957 | F | 5′-CGCGGATCCATGGACCAAAAGTGGATTC-3′ (BamH I) |
| CD8-α | | R | 5′-CCCAAGCTTTGTGTTAGTGTTGCCCTTAC-3′ (Hind III) |
| CD8-β | AB643633 | F | 5′-CCGGAATTCATCCTCCTGCAAGAACCCAT-3′ (EcoR I) |
| CD8-β | | R | 5′-CCCAAGCTTGACAGTCCCAGGCTTCCATAC −3′ (Hind III) |
| IgM | AF226284 | F | 5′-ACAAAAGCCATTGTGAGATCCA-3′ |
| IgM | | R | 5′-TTGACCAGGTTGGTTGTTTCAG-3′ |
| IgT | KX174301 | F | 5′-TAATTGTTCAGTAACTCATGCCG-3′ |
| IgT | | R | 5′-GATTGAAGTGTTCCTATGCGTCT-3′ |
| TCRα | AB053229.1 | F | 5′-CCAGACAAAACTGAAGAG-3′ |
| TCRα | | R | 5′- GAGTTCATAGTAGGACGG-3′ |
| TCRβ | AB053228.1 | F | 5′-CCCCACGACATCTCAAG-3′ |
| TCRβ | | R | 5′- GTCTGGACCTTGTTCACC-3′ |
| CD3ε | AB081751.1 | F | 5′-ATATGATTAAAGTCACATCAGG-3′ |
| CD3ε | | R | 5′-ATTGTGGTTAATGACATCATACG-3′ |
| β-actin | HQ386788.1 | F | 5′-AGAGCAAGAGAGGCATCCTGAC-3′ |
| β-actin | | R | 5′-CGATGGGTGATGACCTGTCC-3′ |