Fish and Shellfish Immunology 91 (2019) 216–222

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

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

Full length article

Development of monoclonal antibody against IgM of large yellow croaker (*Larimichthys crocea*) and characterization of IgM$^+$ B cells

Yupeng Huang[a,b,1], Xiaoqin Yuan[a,c,1], Pengfei Mu[b], Qiuhua Li[b], Jingqun Ao[b,**], Xinhua Chen[a,b,c,d,*]

[a] Key Laboratory of Marine Biotechnology of Fujian Province, Institute of Oceanology, Fujian Agriculture and Forestry University, Fuzhou, 350002, China
[b] Key Laboratory of Marine Biogenetic Resources, Third Institute of Oceanography, State Oceanic Administration, Xiamen, 361005, China
[c] College of Life Sciences, Fujian Agriculture and Forestry University, Fuzhou, 350002, China
[d] Laboratory for Marine Biology and Biotechnology, Qingdao National Laboratory for Marine Science and Technology, Qingdao, 266071, China

ARTICLE INFO

Keywords:
Monoclonal antibody
Immunoglobulin M
Large yellow croaker (*Larimichthys crocea*)
Phagocytosis
IgM$^+$ B cells

ABSTRACT

In the present study, a monoclonal antibody (mAb) against large yellow croaker IgM was produced by immunizing mice with purified large yellow croaker serum IgM. Western blotting showed that this mAb could specifically react with the heavy chain of large yellow croaker serum IgM. Indirect immunofluorescence assay (IFA) analysis suggested that the resulting mouse anti-IgM mAb could recognize membrane-bound IgM (mIgM) molecules of large yellow croaker. This mouse anti-IgM mAb also can be used for sorting of large yellow croaker IgM$^+$ B cells through the magnetic-activated cell sorting (MACS) method, which was further confirmed by RT-PCR analysis of specific marker genes for B cells. Flow cytometry analysis showed that the percentages of IgM$^+$ B cells in head kidney, spleen and peripheral blood lymphocytes were 29.00 ± 1.58%, 33.00 ± 1.64%, and 16.50 ± 2.39%, respectively. Additionally, the phagocytosis rates of IgM$^+$ B cells for 0.5 μm beads in head kidney, spleen and peripheral blood were calculated to be 7.56 ± 0.58%, 4.053 ± 0.62% and 23.17 ± 2.26%, respectively, while only 2.36 ± 0.23%, 1.16 ± 0.44% and 6.41 ± 0.45 of IgM$^+$ B cells in these three tissues ingested 1 μm beads. Taken together, our data demonstrated that the mouse anti-IgM mAb produced in this study could be used as a tool to characterize IgM$^+$ B cells and to study functions of IgM in large yellow croaker.

1. Introduction

It is now well accepted that fish possess innate (non-specific) and adaptive (specific) immune defense systems. The innate parameters are at the forefront of immune defense and are crucial factors in disease resistance. The adaptive immune response of fish is commonly delayed but is essential for long-lasting immunity and is a key factor in successful vaccination [1]. Immunoglobulin (Ig), first occurred in the jawless fish, plays an important role in adaptive immunity [2]. It is known that teleosts possess three major classes of Igs, including IgM, IgD and IgT/Z [3]. IgM is the predominant isotype and specialized in systemic immunity. IgT is an immunoglobulin specialized in mucosal immunity and is considered to be the functional equivalent to IgA in mammals and birds [4]. Teleost IgM exists in two physical forms, a membrane-bound form that is attached to the surface of B cell, referred to as the B cell receptor (BCR), and a soluble form that is secreted by plasma cells [5,6]. Teleost IgD has an unclear function, but it has been suggested that it might play a role in immune response as an antigen binding receptor [7].

B cells are key players in adaptive immune responses through the antibody production. Different subsets of B cells have been reported for different fish species, such as IgM$^-$/IgD$^-$/IgT$^+$, IgM$^+$/IgD$^+$/IgT$^-$ and IgM$^-$/IgD$^+$/IgT$^-$ in rainbow trout (*Oncorhynchus mykiss*) [4,8,9] and IgM$^+$/IgD$^-$, IgM$^+$/IgD$^+$ and IgM$^-$/IgD$^+$ in channel catfish (*Ictalurus punctatus*) [7]. Several additional functions of B cells involved in both the adaptive and the innate immune responses have been identified in vertebrate species including fish. In zebrafish, B cells have the capacity to present both soluble and particulate antigens to specific T cells, thus

* Corresponding author. Key Laboratory of Marine Biotechnology of Fujian Province, Institute of Oceanology, Fujian Agriculture and Forestry University, 15 Shangxiadian Road, Fuzhou, Fujian, 350002, China.
** Corresponding author. Key Laboratory of Marine Biogenetic Resources, Third Institute of Oceanography, State Oceanic Administration, 178 university road, Xiamen 361005, China.
E-mail addresses: ajingqun@tio.org.cn (J. Ao), chenxinhua@tio.org.cn (X. Chen).
[1] Yupeng Huang and Xiaoqin Yuan contributed equally to this work.

https://doi.org/10.1016/j.fsi.2019.05.035
Received 28 February 2019; Received in revised form 12 May 2019; Accepted 17 May 2019
Available online 20 May 2019

acting as antigen presenting cells (APCs) [10]. B cells also have innate phagocytic capacity in several fish species such as rainbow trout [9], Atlantic salmon (*Salmo salar L.*) [11], Atlantic cod (*Gadus morhua L.*) [11], lumpfish (*Cyclopterus lumpus L.*) [12], half-smooth tongue sole (*Cynoglossus semilaevis*) [13], sea bass (*Lateolabrax japonicus*) [14] and turbot (*Scophthalmus maximus*) [15]. Moreover, the membrane-bound IgM $(mIgM)^+$ phagocytic lymphocytes are found to be the main fraction of the phagocytic leukocytes in Atlantic salmon and lumpfish [11,12]. Additionally, fish B cells also play an important role in the early stages of pathogenic antigen recognition and in initiation of the inflammatory responses by secretion of pro-inflammatory cytokines [16,17]. Therefore comprehensive studies of the role of B cells in teleost fish will be helpful to elucidate the functions of B cells within both the adaptive and the innate immune systems. As an initial step, identification of B cells in more fish species is needed.

The large yellow croaker (*Larimichthys crocea*) is an economically important marine fish cultured in China [18]. With rapid development of the large yellow croaker culture industry, the infectious diseases caused by bacteria, parasites, and viruses [19–21], are becoming more and more severe, resulting in huge economic losses. However, information about the functions of immune system in this species is limited, which hinders the establishment of effective disease control ways. In the present study, we prepared a monoclonal antibody (mAb) against large yellow croaker IgM by immunizing mice with the purified serum IgM. The specific mAb was screened and characterized by indirect enzyme-linked immunosorbent assay (ELISA), Western blotting, and indirect immunofluorescence assay (IFA). Then IgM^+ B cells were sorted with the prepared mAb by magnetic-activated cell sorting (MACS) method and identified by specific marker genes for B cells. Furthermore, the percentages of IgM^+B cells in the head kidney, spleen and peripheral blood lymphocytes and their phagocytic ability were analyzed. These results will facilitate the further understanding of characteristics and functions of B cells in large yellow croaker.

2. Materials and methods

2.1. Purification of large yellow croaker IgM

Large yellow croakers (weight: 400 ± 32 g) were purchased from Mari-culture farm at Ningde, Fujian, China. Blood was drawn from the caudal vein of fish using 10 mL syringe with 21 gauge needle, and stored at 4 °C overnight after clotting at room temperature. The serum was collected after 3000 g centrifugation at 4 °C, and stored at – 20 °C. Large yellow croaker serum IgM was purified by a combination of affinity chromatography with a protein-A affinity column (GE Healthcare) and gel-filtration chromatography according to the previously described method with a modulation [22].The purity and molecular weight of the purified IgM were examined by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained by silver nitrate.

2.2. Production of monoclonal antibody

MAb against large yellow croaker IgM was produced as previously described [23] Briefly, three female BALb/c mice were immunized each by intraperitoneal injection with 100 μg of purified IgM in a total volume of 0.2 mL with an equivalent volume of Freund's complete adjuvant (Sigma, St. Louis, Mo, USA). After two weeks, a similar immunization was performed using Freund's incomplete adjuvant (Sigma, St. Louis,Mo, USA) instead of Freund's complete adjuvant. Then two booster immunizations were carried out by tail vein at 7-day intervals with 100 μg purified IgM. At four days after final immunization, the spleen cells from the immunized mouse were collected and fused with myeloma cells, P3-NSl/4.1 cells, using 50% polyethylene glycol 4000 (BDH, Poole, GB). The fused cells were dispensed into sterile 96-well microtiter plates (Flow Labs, MacLean, VA) and progressively diluted using DMEM basic medium containing hypoxanthine, aminopterin, and thymidine. Hybridomas were primarily screened by indirect ELISA. Positive hybridomas were cloned three times by the limited dilution method. Finally, the mAb against IgM was characterized by Western blotting and IFA.

2.3. Isolation of leukocytes

Leukocytes were isolated from head kidney, spleen and peripheral blood of large yellow croakers as reported previously [24,25]. Briefly, large yellow croaker head kidney and spleen tissues were sieved through a 70-μm nylon mesh (BD, USA) to get single cell suspension, respectively. Each cell suspension was then washed with ice-cold L-15 medium containing 2% FBS, 10 IU/mL heparin, 200 IU/mL penicillin (Gibco, USA) and 200 mg/mL streptomycin (Sigma, USA). Then the single cell suspension was loaded onto 51/40% discontinuous Percoll density gradients (GE Healthcare) and separated via centrifugation at $650 \times g$ for 30 min at 4 °C. Leukocytes lying at the gradient interface were collected and washed with ice-cold L-15 medium. The head kidney and spleen leukocytes obtained were stored in ice-cold L-15 medium for future uses, respectively. To isolate peripheral blood leukocytes, blood was sampled from the caudal vein of large yellow croakers and diluted by PBS containing 10 U/mL heparin. The blood sample was then loaded onto 51/40% discontinuous Percoll density gradients for isolation of peripheral blood leukocytes as above.

2.4. Indirect ELISA

The purified IgM was diluted to a concentration of 10 μg/mL in carbonate-bicarbonate buffer (50 mM, pH 9.6). Each well of the 96-well plate was covered with 100 μL diluted IgM overnight at 4 °C followed by washing with PBST (0.2% Tween-20 in PBS) and blocking with 1% skim milk in PBST for 1 h at 37 °C. After washing three times with PBST, 100 μL of candidate hybridomas culture supernatant per well was added to three parallel wells and subsequently incubated for 1 h at 37 °C. Following a further washing, the wells were incubated for 1 h at 37 °C with goat anti-mouse IgG (H + L)-HRP conjugate (1:5000, MultiSciences, Hangzhou, China). Color development was performed with the TMB Substrate solution (Solaibio, Beijing, China). The plates were read at 450 nm with a microplate reader (SpectraMax M3, Molecular Devices, USA). Myeloma culture supernatant instead of hybridomas culture supernatant was used as negative control.

2.5. Indirect immunofluorescence assay (IFA)

The head kidney leukocytes isolated above were seeded in the Confocal Petri dish at a density of 2×10^5 cells/dish. The cells were allowed to settle for 2 h and fixed with 4% paraformaldehyde. Then they were blocked with 5% bovine serum albumin for 30 min followed by washing with PBS. The cells were incubated with mouse anti-IgM mAbs (1:100) for 30 min at 4 °C. After washing three times with PBS, the cells were incubated with goat anti-mouse IgG (H + L) Highly Cross-Adsorbed Secondary Antibody Alexa Fluor 488 conjugate (1:400, Thermo Fisher Scientific, USA) for 30 min at 4 °C,and then stained with 4', 6-diamidino-2-phenylindole(DAPI, 10 mg/mL) according to the instructions of the manufacturer (Sangon, Shanghai, China). The cells were observed under a confocal laser-scanning microscope (CLSM 510; Carl Zeiss).

2.6. Sorting of IgM^+ B cells

In order to maximize cell viability, the magnetic-activated cell sorting (MACS) method was used for sorting of large yellow croaker head kidney IgM^+ B cells as previously described [26]. Briefly, the head kidney leukocytes obtained above (10^7 cells) were blocked with 5% normal goat serum (Solarbio, China) at 4 °C for 15 min, and cells were

resuspended in MACS Running Buffer (Miltenyi Biotec GmbH) containing mouse anti-IgM mAbs conjugated with FITC (1:100) at 4 °C for 30 min, then washed with MACS buffer (2 mM EDTA and 0.5% BSA in PBS). The resulting cell suspensions were incubated with anti- FITC MicroBeads (Miltenyi Biotec) at a MACS buffer/bead ratio of 1/10. The IgM^+ B cells were filtered with a 40 μm strainer and sorted from LS separation columns (Miltenyi Biotec GmbH) according to the manufacturer's instructions. The MACS-sorted IgM^+ B cells were analyzed by flow cytometry and RT-PCR. In RT-PCR analysis, specific markers for B cells [IgM heavy chain (IgH) and light chain (IgL)] and T cells (CD4, CD8, CD3ε, TCR-α, and TCR-δ) were detected. Primer sequences of target genes are listed in Supplementary Table 1.

2.7. Detection of IgM^+ B cells by flow cytometer

Leukocytes isolated from head kidney, spleen and peripheral blood were processed for flow cytometry analysis (FCA) as previously described [25,26]. Cells (10^7 cells each) were resuspended in 100 μL PBS containing 2% BSA and incubated with mouse anti-IgM mAbs (1:100) for 30 min at 4 °C. After washed three times with PBS containing 2% BSA, the cells were incubated with goat anti-Mouse IgG (H + L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 (1:400; Thermo Fisher) for 30 min at 4 °C. The cells then were washed three times with PBS containing 2% BSA. Flow cytometer was used to analyze the cell samples (BD Accuri C6, USA). Normal mouse serum instead of mouse anti-IgM mAbs was used as negative control.

2.8. Detection of phagocytosis of fluorescence microspheres by IgM^+ B cells

The phagocytic capacity of large yellow croaker IgM^+ B cells was evaluated as previously reported [9]. Briefly, leukocytes from head kidney, spleen and peripheral blood (2×10^6 cells each) were incubated with 0.5 μm and 1 μm fluorescence microspheres [red fluorescence (580/605); Thermo Fisher] for 2 h at 28 °C at a cell/bead ratio of 1/10, respectively. Non-ingested microspheres were removed by centrifuging two times at 450 × *g* for 5 min at 4 °C after loading the cell suspensions over a cushion of 2% BSA in PBS. Then, the phagocytic cells were resuspended in L-15 medium and incubated with mouse anti-IgM mAbs (1:100)for 30 min at 4 °C, followed by an incubation with goat anti-Mouse IgG (H + L) Highly Cross-Adsorbed Secondary Antibody (1:400; Thermo Fisher) for 30 min at 4 °C. After washing three times, samples were analyzed by flow cytometer as above. Leukocytes from head kidney, spleen and peripheral blood that were not incubated with microspheres were set as negative control.

To further observe the phagocytosis of fluorescence microspheres by large yellow croaker IgM^+ B cells, the IgM^+ B cells were sorted from head kidney leukocytes (10^7 cells) as above and incubated with 0.5 μm fluorescence microspheres [red fluorescence (580/605)] for 2 h at 28 °C, after washing three times with PBS, the cells were incubated with goat anti-mouse IgG (H + L) Highly Cross-Adsorbed Secondary Antibody Alexa Fluor 488 conjugate (Thermo Fisher Scientific, USA) for 30 min at 4 °C,and then fixed with 4% paraformaldehyde. After washing, the cells were stained with DAPI and observed under confocal laser-scanning microscope.

2.9. Statistical analysis

All data were analyzed using GraphPad Prism 5 software and expressed as mean ± the standard error of the mean (SEM). The data were subjected to analysis of one way ANOVA by using IBM SPSS Statistics 19, and the *P* values smaller than 0.05 were considered statistically significant.

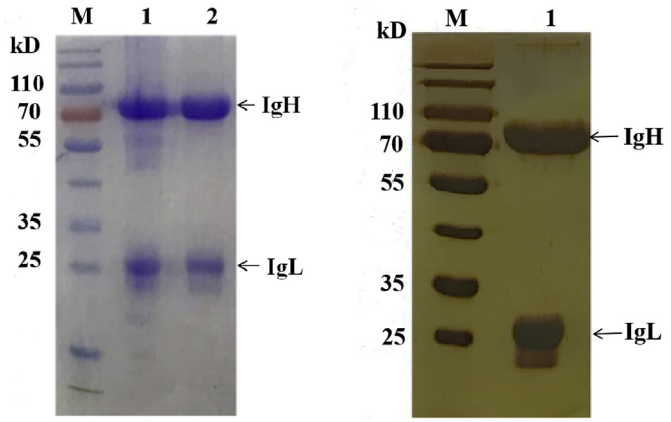

Fig. 1. Purified large yellow croaker serum IgM. The purified serum IgM was analyzed by SDS-PAGE and stained by Coomassie brilliant blue R250 (left panel) and silver nitrate (right panel), respectively. Left panel: M, molecular weight standard; Lane 1, IgM purified by protein-A affinity column; Lane 2, further purified IgM by gel-filtration. Right panel: M, molecular weight standard; Lane 1, purified IgM stained by silver nitrate. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3. Results

3.1. Serum IgM purification

Large yellow croaker serum IgM was purified by a combination of affinity chromatography and gel-filtration chromatography. The SDS-PAGE analysis showed that purified IgM displayed two main bands with the molecule weights of approximately 70 kDa and 27 kDa (Fig. 1), which correspond to the heavy and light chains of IgM, respectively.

3.2. Characterization of mouse anti-IgM monoclonal antibody

A hybridomas (designated as 9G7), which gave strong positive results in contrast to myeloma culture supernatant in indirect ELISA, was screened. This hybridomas strain was then cloned by limiting dilution. Western-blotting analysis showed that resulting mAb 9G7 specifically reacted with the heavy chain of large yellow croaker serum IgM in reducing condition (Fig. 2). Indirect IFA analysis further showed that green fluorescence signals were detected on the membrane of partial leukocytes (Fig. 3), indicating that mAb 9G7 could recognize the mIgM molecules on these leukocytes.

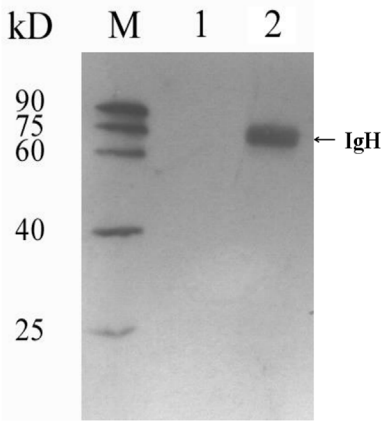

Fig. 2. Western blotting analysis of specificity of mAb against IgM. M: molecular weight standard. Lane 1, negative control; Lane2, purified large yellow croaker serum IgM; Arrow, heavy chain of IgM (IgH, approximately 70 kDa).

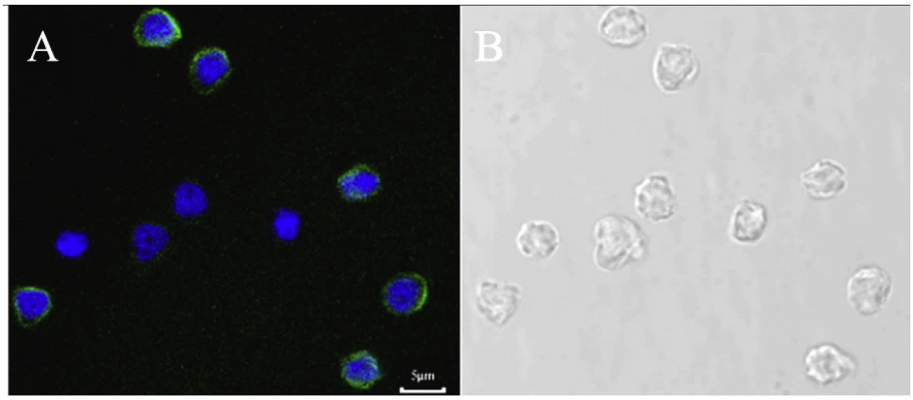

Fig. 3. Immunofluorescence staining of large yellow croaker head kidney leukocytes. Head kidney leukocytes were incubated with mouse anti-IgM mAb 9G7, followed by incubation with goat anti-mouse IgG (H + L) Highly Cross-Adsorbed Secondary Antibody. Myeloma culture supernatant instead of mAb 9G7 was used as negative control. The DAPI staining shows the nuclei of leukocytes. A, immunofluorescence-stained leukocytes with mAb 9G7 under fluorescent field; B, cells were observed under bright field. . (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.3. Sorting of IgM$^+$ B cells

To investigate whether the mouse anti-IgM mAb obtained can be used for sorting of large yellow croaker IgM$^+$ B cells, the MACS method was performed to sort IgM$^+$ B cells from head kidney leukocytes. Then the sorted IgM$^+$ and IgM$^-$ head kidney leucocytes were analyzed by flow cytometer. As shown in Fig. 4 A, the purity of the sorted IgM$^+$ leukocytes was nearly 99%. Meanwhile the IgM$^-$ leucocytes (unlabeled leucocytes) were about 94.3% of outflowing cells (Fig. 4 B). Additionally, the expression of several marker genes was also examined in the sorted IgM$^+$ leukocytes and IgM$^-$ leucocytes by RT-PCR. As expected, IgM$^+$ leukocytes could express IgH and IgL genes, specific markers for B cells, while IgM$^-$ leucocytes express others (Fig. 4 C), further indicating that the sorted cells were large yellow croaker IgM$^+$ B cells.

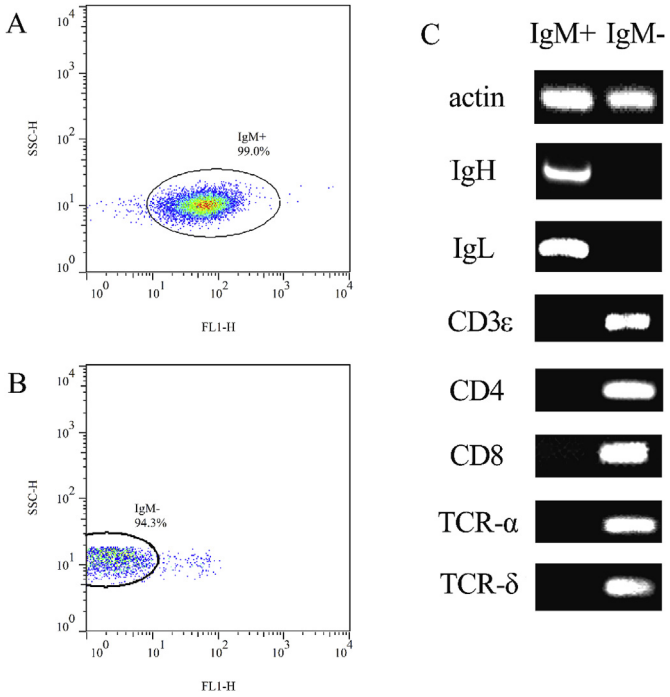

Fig. 4. Flow cytometry analysis and identification of sorted IgM$^+$ B cells. The MACS method was performed to sort IgM$^+$ B cells from head kidney leukocytes. Head kidney leukocytes (10^7 cells) were incubated with mouse anti-IgM mAb conjugated with FITC at 4 °C for 30 min, followed by an incubation with anti-mouse IgG magnetic beads at a MACS buffer/bead ratio of 1/10. The IgM$^+$ B cells were sorted by LS separation columns, and the sorted IgM$^+$ B cells analyzed by flow cytometer. A, sorted IgM$^+$ B cells; B, IgM$^-$ head kidney leucocytes; C, specific marker genes for B cells (IgH and IgL) and T cells (CD4, CD8, CD3ε, TCR-α, and TCR-δ) were detected in the sorted IgM$^+$ B cells and IgM$^-$ leucocytes by RT-PCR.

3.4. Percentages of IgM$^+$ B cells in head kidney, spleen and peripheral blood

To characterize large yellow croaker IgM$^+$ B cells according to their percentages in head kidney, spleen and peripheral blood, the leukocytes from head kidney, spleen and peripheral blood were isolated from ten individual fish and fluorescently strained for FCA. The forward scatter (FSC) and sideward scatter (SSC) parameters represented cell size and granularity, respectively (Fig. 5A, left panel). Based on the separation of the major blood cell lines in the head kidney of the zebrafish [27], in this study the lymphocytes were gated and then the fractions of IgM$^+$ B cells in head kidney lymphocytes (HKLs), spleen lymphocytes (SLs), and peripheral blood lymphocytes (PBLs) were determined (Fig. 5A, right panel). The results showed that the percentages of IgM$^+$ B cells in HKLs, SLs, and PBLs were 29.00 ± 1.58%, 33.00 ± 1.64%, and 16.50 ± 2.39%, respectively (Fig. 5B).

3.5. Phagocytosis of fluorescence microspheres by IgM$^+$ B cells

After incubation with fluorescent microsphere beads of different sizes, phagocytosis rates of IgM$^+$ B cells in head kidney, spleen and peripheral blood were analyzed by flow cytometry. The lymphocytes in four quadrants, Q1, Q2, Q3, and Q4 of fluorescent dot plots represented phagocytic IgM$^-$ lymphocytes, phagocytic IgM$^+$ B cells, non-phagocytic IgM$^+$ B cells, and non-phagocytic IgM$^-$ lymphocytes, respectively (Fig. 6A, upper panel: 0.5 μm fluorescent beads; lower panel: 1 μm fluorescent beads). The phagocytosis rates of IgM$^+$ B cells for 0.5 μm beads in head kidney, spleen and peripheral blood were calculated to be 7.56 ± 0.58%, 4.053 ± 0.62%, and 23.17 ± 2.26%, respectively, while only 2.36 ± 0.23%, 1.16 ± 0.44%, and 6.41 ± 0.45 of IgM$^+$ B cells in these three tissues ingested 1 μm beads (Fig. 6B), indicating that the phagocytic capacity of IgM$^+$ B cells was higher for 0.5 μm beads than for 1 μm beads. IgM$^+$ B cells sorted from head kidney leukocytes were incubated with 0.5 μm beads, and then observed by confocal laser-scanning microscope (Fig. 6C). The IgM$^+$ B cell membrane was labeled with green fluorescence (Fig. 6C, b). IgM$^+$ B cells showed endocytic activity against 0.5 μm fluorescent microspheres(Fig. 6C, d) with red fluorescence (Fig. 6C, c).

4. Discussion.

Among three types of Igs in teleost fish, IgM is the predominant isotype and plays a key role in systemic immunity [6]. MAbs against IgM have been developed in several fish species, such as European sea bass (*Dicentrarchus labrax*) [28], flounder (*Paralichthys olivaceus*) [29], rainbow trout [9], turbot (*Scophthalmus maximus*) [23], half-smooth tongue sole [13], and sea bass [14], which facilitates the identification of IgM$^+$ lymphocytes. In the previous study, we cloned large yellow croaker IgM heavy chain and light chain genes and found that their expression levels were quickly increased upon the stimulation of poly

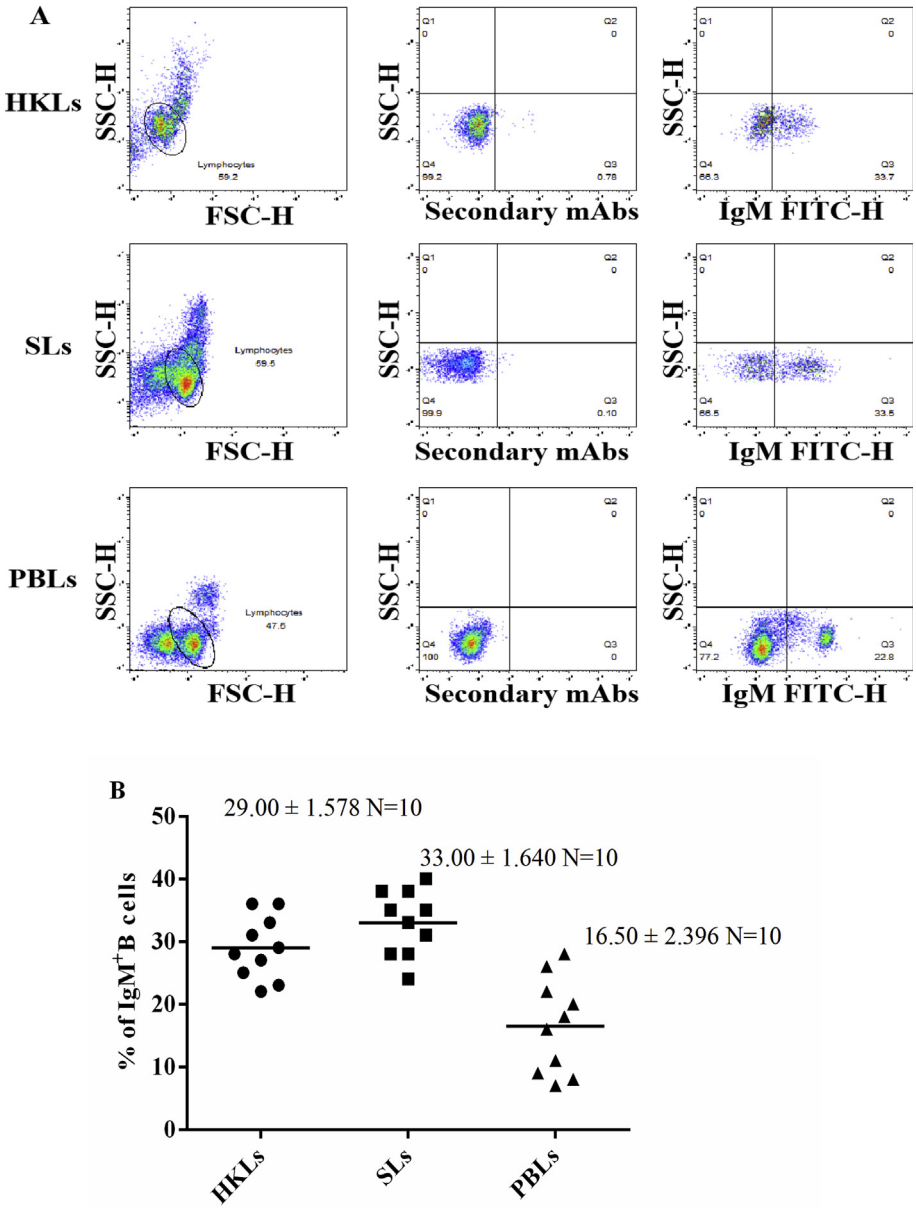

Fig. 5. Percentages of IgM$^+$ B cells in head kidney, spleen and peripheral blood lymphocytes.
IgM+ B cells in large yellow croaker HKLs, SLs, and PBLs. A, Flow cytometry detection of IgM+ B cells in HKLs, SLs, and PBLs. FSC area (FSC–H)/SSC area (SSC–H) analyses are shown in the left panel, and green gate represents lymphocytes; middle panels represent the leukocytes treated with normal mouse serum instead of mouse anti-IgM mAbs; right panels represent the leukocytes stained with mouse anti-IgM mAbs. B, Percentages of IgM+ B cells in HKLs, SLs, and PBLs. Data represent mean ± SEM of percentages of IgM+ B cells from ten fish.

(I:C) or bacterial vaccine, suggesting a possible involvement of the IgM in the early phase of immune response [30,31]. In the present study, we successfully produced a mAb 9G7 against heavy chain of large yellow croaker serum IgM (Fig. 2). In the next screening, another mAb was obtained, which was also against heavy chain of large yellow croaker IgM (data not shown). These results further support that immunogenicity of heavy chain of fish IgMs is much stronger than that of light chain [13]. Western-blotting and IFA analyses showed that the mAb 9G7 specifically reacted with serum IgM (heavy chain) and mIgM on the B cells (Figs. 2 and 3), indicating that the resulting mAbs can recognize both soluble IgM and mIgM on the B cells.

Then, this mAb was used for sorting of large yellow croaker IgM$^+$ B cells by the MACS method. The result showed that this mouse anti-IgM mAb could label large yellow croaker IgM$^+$ B cells and the sorted IgM$^+$ B cells had a purity of nearly 99% (Fig. 4 A). This was further confirmed by RT-PCR analysis, where the sorted IgM$^+$ B cells could express IgH and IgL genes, specific markers for B cells, while IgM$^-$ leucocytes only express others (Fig. 4 C). These results indicated that the mAb obtained here can be used for sorting of large yellow croaker IgM$^+$ B cells.

Accumulating data showed that the levels of IgM$^+$ B cells or IgM$^+$ lymphocytes varied among fish species [28,29]. In this study, the percentages of large yellow croaker IgM$^+$ B cells in HKLs, SLs, and PBLs were approximately 29.00%, 33.00%, and 16.50%, respectively, with the lowest rate of IgM$^+$ B cells observed in PBLs (Fig. 5B). These results were different from those obtained in other fish species, where the levels of IgM$^+$ B cells in peripheral blood were the highest among the immune-related tissues, such as the percentage of mIgM + lymphocytes in peripheral blood was higher than those of spleen and pronephros in half-smooth tongue sole, sea bass and turbot [13–15]. The phagocytic IgM$^+$ B cells exist in teleost fish such as rainbow trout, Atlantic salmon, Atlantic cod, lumpfish, half-smooth tongue sole and turbot [9,11–13,15], amphibians [9] and mammals [32]. Human B cells

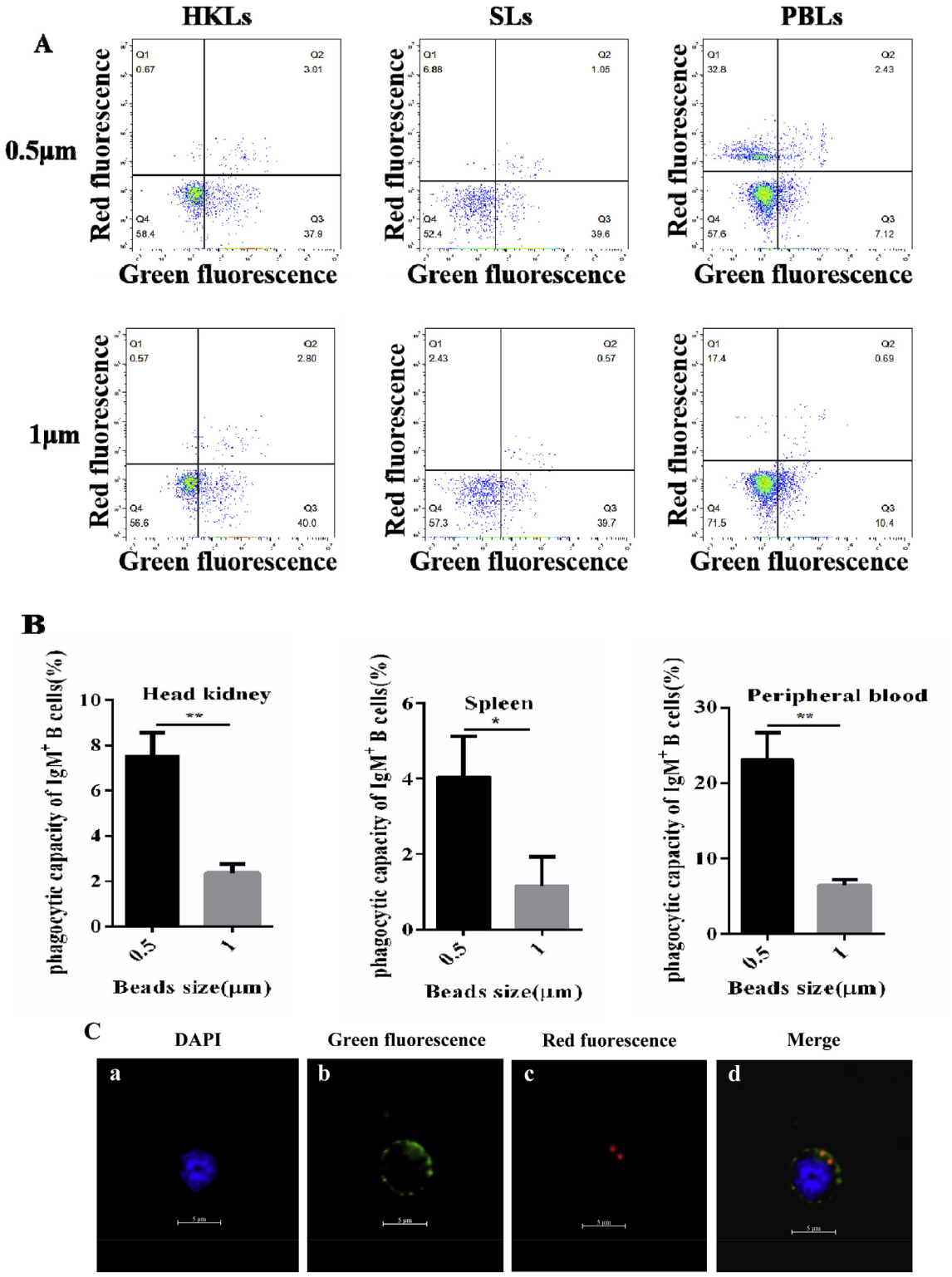

Fig. 6. Phagocytosis of fluorescence microspheres by IgM^+ B cells.

Phagocytosis rates of large yellow croakers IgM+ B cells for fluorescent microsphere beads of different sizes. A, Leukocytes from head kidney, spleen and peripheral blood were incubated with 0.5 μm and 1 μm beads, respectively, and then treated with anti-IgM mAb and goat anti-Mouse IgG (H + L) Highly Cross-Adsorbed Secondary Antibody. The cells were subjected to FCA for phagocytic capacity of IgM+ B cells from a representative individual. Q1, Q2, Q3 and Q4 of fluorescent dot plots represent phagocytic IgM− lymphocytes, phagocytic IgM+ B cells, non-phagocytic IgM+ B, and non-phagocytic IgM− lymphocytes, respectively. B, Rates of IgM+ B cells ingesting beads with different sizes. Data represent mean ± SEM of ten fish. Asterisks indicate the statistical significance of phagocytic rates between 0.5 μm and 1 μm fluorescent microsphere beads in each tissue (*$p < 0.05$, **$P < 0.01$). C, Detection of endocytic IgM+ B cells by confocal laser-scanning microscope. IgM+ B cells sorted from head kidney leukocytes (b) were incubated with 0.5 μm beads (c). The cells were stained with DAPI and subjected to fluorescence inverted microscopy(a). d, a merged image of a, b, and c((Bars = 5 μm).

mainly play a role in acquired immunity, and only a small part of mIgM$^+$ lymphocytes show phagocytic capacity [32]. In the present study, we demonstrated that large yellow croaker IgM$^+$ B cells could phagocytose 0.5 μm or 1 μm fluorescent microsphere beads, indicating that large yellow croaker IgM$^+$ B cells also had phagocytic capacity (Fig. 6). Moreover, the phagocytic capacity of IgM$^+$ B cells was higher for 0.5 μm beads than for 1 μm beads (Fig. 6), which was similar with the previous report that the uptake capacity of rainbow trout and turbot IgM$^+$ B cells was also higher for 0.5 μm beads than for 1 μm beads [9,15]. These data suggested that particle-uptake of fish IgM$^+$ B cells was particle-size dependent. The percentage of phagocytic IgM$^+$ B cells was the highest in the peripheral blood of large yellow croaker, as found in other fish species, including rainbow trout and turbot [9,15]. Approximately 23% of large yellow croaker IgM$^+$ B cells in peripheral blood were capable to ingest 0.5 μm beads (Fig. 6), which was similar with that of turbot IgM$^+$ B cells (21%) [15], but lower than that of rainbow trout IgM$^+$ B cells (82%) [9]. In addition, we found that the IgM$^-$ cell population also has phagocytic capacity, which was similar with the results observed in rainbow trout and turbot [9,15]. The possible reason is that the IgM$^-$ cell population includes some phagocytes such as monocytes/macrophages. This difference in ingestion ability by IgM$^+$ B cells suggests a different function for IgM$^+$ B cells as nonprofessional phagocytes in different fish species. Additionally, such difference may be due to the fish status as well as the experimental operations varied in different laboratories. Further investigations are needed to elucidate the pathway by which large yellow croaker IgM$^+$ B cells ingest the exogenous particles.

In conclusion, we successfully produced a mAb 9G7 against large yellow croaker IgM, which can be used for sorting of large yellow croaker IgM$^+$ B cells. Moreover, we also found that large yellow croaker IgM$^+$ B cells had phagocytic capacity and their phagocytosis rates varied in different tissues. These results therefore facilitate the further understanding of characteristics and functions of B cells in large yellow croaker.

Acknowledgements

The work was supported by grants from the National Key R&D Program of China (National Natural Science Foundation of China2018YFD0900503), National Natural Science Foundation of China (U1605211 and 31772874), China Agricultural Research System (CARS-47) and 13th Five-year Plan on Fuzhou Marine Economic Innovation and Development Demonstration City Project.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.05.035.

References

[1] C.J. Secombes, T. Wang, 1 – the innate and adaptive immune system of fish, Infect. Disease. Aquaculture 14 (2012) 3–68.
[2] M.F. Flajnik, Comparative analyses of immunoglobulin genes: surprises and portents, Nat. Rev. Immunol. 2 (2002) 688–698.
[3] S. Fillatreau, A. Six, S. Magadan, R. Castro, J.Q. Sunyer, P. Boudinot, The astonishing diversity of Ig classes and B cell repertoires in teleost fish, Front. Immunol. 28 (2013) 6–19.
[4] Y.A. Zhang, I. Salinas, J.O. Sunyer, Recent findings on the structure and function of teleost IgT, Fish Shellfish Immunol. 31 (2011) 627–634.
[5] A. Zapata, B. Diez, T. Cejalvo, C. Gutierrez-de Frias, A. Cortes, Ontogeny of the immune system of fish, Fish Shellfish Immunol. 20 (2) (2006) 126–136.
[6] E. Randelli, F. Buonocore, G. Scapigliati, Cell markers and determinants in fish immunology, Fish Shellfish Immunol. 25 (2008) 326–340.
[7] E.S. Edholm, E. Bengten, J.L. Stafford, M. Sahoo, E.B. Taylor, N.W. Miller, et al., Identification of two IgD(+) B cell populations in channel catfish, *Ictalurus punctatus*, J. Immunol. 185 (2010) 4082–4094.
[8] R. Castro, E. Bromage, B. Abós, J. Pignatelli, A.G. Granja, A. Luque, et al., CCR7 is mainly expressed in teleost gills, where it defines an IgD$^+$IgM$^-$ B lymphocyte subset, J. k. 192 (2014) 1257–1262.
[9] J. Li, D.R. Barreda, Y.A. Zhang, H. Boshra, A.E. Gelman, S.B. LaPatra, et al., B lymphocytes from early vertebrates have potent phagocytic and microbicidal abilities, Nat. Immunol. 7 (2006) 1116–1124.
[10] L.Y. Zhu, A.F. Lin, T. Shao, L. Nie, W.R. Dong, L.X. Xiang, et al., B cells in teleost fish act as pivotal initiating APCs in priming adaptive immunity: an evolutionary perspective on the origin of the B-1 cell subset and B7 molecules, J. Immunol. 192 (2014) 2699–2714.
[11] H.S. Overland, E.F. Pettersen, A. Ronneseth, H.I. Wergeland, Phagocytosis by B-cells and neutrophils in Atlantic salmon (*Salmo salar L.*) and Atlantic cod (*Gadus morhua L.*), Fish Shellfish Immunol. 28 (2010) 193–204.
[12] A. Ronneseth, D.B. Ghebretnsae, H.I. Wergeland, G.T. Haugland, Functional characterization of IgM+ B cells and adaptive immunity in lumpfish (*Cyclopterus lumpus L.*), Dev. Comp. Immunol. 52 (2015) 132–143.
[13] S. Yang, X.Q. Tang, X.Z. Sheng, J. Xing, W.B. Zhan, Development of monoclonal antibodies against IgM of half-smooth tongue sole (*Cynoglossus semilaevis*) and analysis of phagocytosis of fluorescence microspheres by mIgM+ lymphocytes, Fish Shellfish Immunol. 66 (2017) 280–288.
[14] S. Yang, X.Q. Tang, X.Z. Sheng, J. Xing, W.B. Zhan, Development of monoclonal antibodies against IgM of sea bass (*Lateolabrax japonicus*) and analysis of phagocytosis by mIgM+ lymphocytes, Fish Shellfish Immunol. 78 (2018) 372–382.
[15] Y.Q. Li, L. Sun, J. Li, Internalization of large particles by turbot (*Scophthalmus maximus*) IgM$^+$ B cells mainly depends on macropinocytosis, Dev. Comp. Immunol. 82 (2018) 31–38.
[16] R. Castro, S. Martinez-Alonso, U. Fischer, N.A. Haro, V. Soto-Lampe, T. Wang, C.J. Secombes, N. Lorenzen, E. Lorenzen, C. Tafalla, DNA vaccination against a fish rhabdovirus promotes an early chemokine-related recruitment of B cells to the muscle, Vaccine 32 (10) (2014) 1160–1168.
[17] R. Castro, B. Abós, L. González, A.G. Granja, C. Tafalla, Expansion and differentiation of IgM+ B cells in the rainbow trout peritoneal cavity in response to different antigens, Dev. Comp. Immunol. 70 (2017) 119–127.
[18] Y.N. Mu, M.Y. Li, F. Ding, Y. Ding, J.Q. Ao, S.N. Hu, X.H. Chen, De novo characterization of the spleen transcriptome of the large yellow croaker (*Pseudosciaena crocea*) and analysis of the immune relevant genes and pathways involved in the antiviral response, PLoS One 9 (2014) e97471.
[19] M.B.K. Owen, H. Ni, G. Wang, X. Wu, A forecasting model for bacterial disease of cage cultured large yellow croaker(*Pseudosciaena crocea*)based on grey system theory, J. Fish. China 37 (2013) 920.
[20] Y.N. Mu, J.Y. Huo, Y.Y. Guan, D.D. Fan, X.X. Xiao, J.G. Wei, Q.H. Li, et al., An improved genome assembly for Larimichthys crocea reveals hepcidin gene expansion with diversified regulation and function, Nat. Commun. Biol 195 (2018) 1–12.
[21] X.H. Chen, K.B. Lin, X.W. Wang, Outbreaks of an iridovirus disease in maricultured large yellow croaker, *Larimichthys crocea* (Richardson), in China, J. Fish Dis. 26 (2003) 615–619.
[22] Q.P. Yan, Y.F. Han, T.X. Gao, Z.X. Zhuang, X.R. Wang, Purification of serum IgM from large yellow croaker*(Pseudosciaena crocea)* and preparation of rabbit sera anti-IgM, J. Fish. Sci. China 133 (2006) 475–479.
[23] W. Zhang, X.Q. Tang, X.Z. Sheng, J. Xing, W.B. Zhan, Development and characterization of monoclonal antibodies to serum IgM of turbot(*Scophthalmus maximus*), P. Ocean. Univ.Chin. 45 (2016) 967–968.
[24] K.Q. Mao, W. Chen, J.Q. Ao, X.H. Chen, Identification of two IL-4/13 homologues in large yellow croaker (Larimichthys crocea) revealed their similar roles in inducing alternative activation of monocytes/macrophages, Fish Shellfish Immunol. 80 (2018) 180–190.
[25] P.F. Mu, Y.H. Wang, J.Q. Ao, C.X. Ai, X.H. Chen, Molecular cloning and bioactivity of an IL-2 homologue in large yellow croaker (*Larimichthys crocea*), Fish Shellfish Immunol. 81 (2018) 309–317.
[26] G. Welzel, D. Seitz, S. Schuster, Magnetic-activated cell sorting (MACS) can be used as a large-scale method for establishing zebrafish neuronal cell cultures, Sci. Rep. 5 (2015) 7959.
[27] D. Traver, B.H. Paw, K.D. Poss, W.T. Penberthy, S. Lin, L. Zon, Transplantation and in vivo imaging of multilineage engraftment in zebrafish bloodless mutants, Nat. Immunol. 4 (2003) 1238–1246.
[28] N.M.S. DosSantos, N. Taverne, A.J. TaverneThiele, M. DeSousa, J.H.W.M. Rombout, Characterisation of monoclonal antibodies specific for sea bass (*Dicentrarchus labrax L.*) IgM indicates the existence of B cell subpopulations, Fish Shellfish Immunol. 3 (1996) 175–191.
[29] 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.
[30] C. Tian, X.H. Chen, J.Q. Ao, The up-regulation of large yellow croaker secretory IgM heavy chain at early phase of immune response, Fish. Physiol. Biol. 36 (2010) 483–490.
[31] C. Tian, Y. Ding, J.Q. Ao, X.H. Chen, Three isotypes of immunoglobulin light chains in large yellow croaker, Pseudosciaena crocea: molecular cloning, characterization, and expression analysis, Fish Shellfish Immunol. 30 (2011) 1249–1256.
[32] Q. Zhu, M. Zhang, M. Shi, Y. Liu, Q. Zhao, W.J. Wang, et al., Human B cells have an active phagocytic capability and undergo immune activation upon phagocytosis of Mycobacterium tuberculosis, Immunobiology 221 (2016) 558–567.