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Macropinocytosis-dependent endocytosis of Japanese flounder IgM$^+$ B cells and its regulation by CD22

Yi-qun Li[a,b,c], Li Sun[a,b,**], Jun Li[b,d,*]

[a] CAS Key Laboratory of Experimental Marine Biology, Institute of Oceanology, Center for Ocean Mega-Science, Chinese Academy of Sciences, Qingdao, China
[b] Laboratory for Marine Biology & Biotechnology and Laboratory for Marine Fisheries Sciences and Food Production Processes, Qingdao National Laboratory for Marine Science and Technology, Qingdao, 266071, China
[c] University of Chinese Academy of Sciences, Beijing, China
[d] School of Biological Sciences, Lake Superior State University, Sault Ste. Marie, MI, 49783, USA

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ABSTRACT

B cells in fish are proven to be endocytic and have a great contribution to innate immunity like phagocytosis. In this study, the endocytic capacity and the corresponding internalization pathways of IgM$^+$ B cells in Japanese flounder *(Paralichthys olivaceus)* were investigated. The results showed that IgM$^+$ B cells in peripheral blood leukocytes (PBL) and splenic leukocytes (SL) exhibited different abilities to ingest 0.5 μm and 1 μm latex beads through macropinocytosis-dependent endocytic pathway. Japanese flounder CD22 (PoCD22) co-stimulatory signals were identified to be essential for the innate immune responses in B cells. Most of IgM$^+$ B cells and some IgM$^-$ cells were demonstrated to be PoCD22 positive. When PoCD22 was blocked by antibody, the endocytic activities and reactive oxygen species (ROS) activities of SL IgM$^+$ B cells were significantly increased, while the endocytic and ROS activities of PBL IgM$^+$ B cells were significant decreased. These results collectively suggest that Japanese flounder IgM$^+$ B cells are able to employ macropinocytosis-dependent endocytic pathway, which is under the regulation of CD22.

1. Introduction

It is well known that B cells in teleost fish have endocytic capacity, and they can phagocytose particular antigens and induce phagolysosome formation and serial downstream degradative activities [1]. Endocytic B cells have so far been identified in many fish species, such as rainbow trout, zebrafish, lumpfish (*Cyclopterus lumpus* L.), half-smooth tongue sole (*Cynoglossus semilaevis*), Atlantic salmon (*Salmo salar* L.), Atlantic cod (*Gadus morhua* L.) and turbot (*Scophthalmus maximus*) [1–7]. So far, three types of immunoglobulins (IgM, IgT, and IgD) have been reported in teleost fish, and IgM, which consists of membrane IgM (B cell receptor, BCR) or soluble IgM, has been demonstrated as the most predominant isotype in teleost fish [8,9]. Membrane IgM$^+$ B cells are the majority B cells in teleost fish as have been demonstrated in salmon and rainbow trout [1,3].

Several endocytosis pathways, such as clathrin-dependent endocytosis, caveolin-dependent endocytosis, macropinocytosis and phagocytosis, have been reported to be involved in the process of particulate antigen internalization [10–13]. For example, human Raji B cells could ingest non-specific bacteria mainly through the pathway of macropinocytosis [14]. Similarly, macropinocytosis pathway was also involved in the internalization of large particles by turbot IgM$^+$ B cells [7]. However, clathrin- and caveolin-dependent pathways, rather than micropinocytosis, were used in IgM$^-$ cells to mediate the endocytosis of large particles [7]. Such specific pathway mediated endocytosis can be inhibited by related pharmacological compounds. Previous studies showed that clathrin-dependent endocytosis can be blocked with CPZ and dynasore; caveolin-dependent endocytosis can be inhibited by M-β-CD and nystatin; and macropinocytosis can be down-regulated by IPA-3 and NSC23766 [15]. Thus far, the endocytic pathways and related regulatory mechanisms involved in the particle internalizing process of phagocytic B cells in Japanese flounder *(Paralichthys olivaceus)* are not fully described yet.

B cell receptor (BCR) and B cell-associated receptors are essential for B cell activation through specific antigen recognization and signal transduction [16,17]. CD22, a B cell-associated receptor, can bind to alpha 2, 6-galactose-linked sialic acids and provide a co-stimulatory signal for activation of B cell. CD22 has been found to distribute

* Corresponding author. School of Biological Sciences, Lake Superior State University, 650 W. Easterday Ave. Sault Ste. Marie, MI, 49783, USA.
** Corresponding author. Institute of Oceanology, Chinese Academy of Sciences, 7 Nanhai Road, Qingdao, 266071, China.
E-mail addresses: lsun@qdio.ac.cn (L. Sun), jli@lssu.edu (J. Li).

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throughout the body and play vital roles such as inhibition of BCR signaling via recruitment of SHP-1 phosphatase, as well as facilitation of adhesion between B cells and other type of cells [18]. In mammals, CD22 has been demonstrated as a negative regulator for antigen receptor signaling, and its onset of expression in mature B cells may serve to raise the threshold of antigen concentration required for B cell activation [19]. However, a positive signaling role for CD22 has also been observed for stimulating human B cell proliferation in the presence of antigen [20]. In our previous study, tongue sole CD22 was found to play an inhibitory role in peripheral blood leukocytes (PBL) activation [21], however, its potential effect on the activation of B cells remains to be investigated.

In the present study, we investigated the endocytic activity and pathway of IgM^+ B cells, as well as the regulating function of CD22 in B cell activation in Japanese flounder (*Paralichthys olivaceus*). Our results indicated that IgM^+ B cells, in both PBL and splenic leukocytes (SL), employed micropinocytosis-pathway to mediate the endocytosis of large particles in Japanese flounder, and CD22 showed different effects on the activation of IgM^+ B cells in PBL and SL.

2. Materials and methods

2.1. Fish

Japanese flounder (*Paralichthys olivaceus*), average 22 cm in length and 600 g in weight, were obtained from a commercial fish farm in Shandong Province, China and maintained at 19–20 °C in aerated seawater. Before experiment, fish were acclimatized to laboratory conditions for two weeks and confirmed to be absent of specific bacterial pathogens as previously described [21].

2.2. Leukocyte isolation

PBL preparation was performed based on previous report [22]. Blood was collected from the caudal vein of Japanese flounder after the fish were euthanized by being immersed in tricaine methanesulfonate solution (Sigma-Aldrich, St. Louis, USA) at the dose of 0.1 g/L in sterilized seawater. Blood was diluted immediately 1:4 with L-15 culture medium (Jinuo, Hangzhou, China) supplemented with 10 units/mL heparin (Solarbio, Beijing, China). Spleen was then collected and placed into a 50 mL test tube containing 30 mL of L-15 culture medium. The tissue was processed by being passed through a 100 μm nylon Falcon cell strainer (BD Falcon, Lexington, KY, USA). All cell suspensions were placed onto a 35/58% discontinuous Percoll (GE Healthcare) density gradients and centrifuged at 400 × *g* for 30 min. The PBL or SL from the interface layer were collected and washed twice with L-15 medium respectively (Gibco, Carlsbad, USA), and then re-suspended in L-15 culture medium containing 4% calf serum (Gibco, Carlsbad, USA), 100 U/mL penicillin (Solarbio, Beijing, China), 100 μg/mL streptomycin (Solarbio, Beijing, China), and 10 units/mL heparin.

2.3. Phagocytosis assay by flow cytometry

The phagocytic capability of Japanese flounder PBL and SL was evaluated as previously described [1]. Briefly, PBL or SL (1×10^6 cells/mL) were incubated with yellow green microsphere beads (Polysciences, Warrington, PA, USA, Fluoresbrite R Yellow Green, λ_{ex}, 445 nm; λ_{em}, 500 nm) of 0.5 μm or 1 μm in diameter at a cell/beads ratio of 1/10 for 4 h at 20 °C respectively. Non-ingested beads were removed by centrifuging (100 × *g* for 10 min at 4 °C) the cell suspension over a cushion of 3% BSA (Aikerbo, Qingdao, China) in PBS supplemented with 4.5% D-glucose (Sigma-Aldrich, St. Louis, USA). Then the phagocytic cells were resuspended in L-15 culture medium and incubated with 5 μg/mL mouse-anti-Japanese flounder IgM monoclonal antibody (mAb) (Aquatic Diagnostics Ltd, Stirling, Scotland) for 1 h at 20 °C, followed by staining with Phycoerythrin (PE)-conjugated goat anti-mouse IgG (Thermo Fisher Scientific, MA, USA) for 1 h at 20 °C. After washing, the phagocytosis of PBL or SL cells were determined by a FACScan flow cytometer (BD Biosciences, USA), and following data analysis by FlowJo.

2.4. Effect of endocytosis inhibitors on leukocyte uptake of microspheres

Microsphere uptake was performed as reported previously [7]. PBL or SL (1×10^6 cells/mL) were pretreated with various inhibitors, i.e., 20 μM chlorpromazine (CPZ) (Selleck, USA), 15 μM dynasore (Selleck, USA), 1 mM methy-β-clodextrin (M-β-CD) (Sigma-Aldrich, St. Louis, USA), 100 μM nystatin (Selleck, USA), 40 μM IPA-3 (Selleck, USA), 100 μM NSC23766 (Selleck, USA), in L-15 medium for 2 h at 20 °C, respectively. The pre-treated PBL or SL were then incubated with microspheres (size 0.5 μm or 1 μm) for 4 h or 2 h at 20 °C. After being washed three times with PBS, the PBL or SL were fixed with 4% paraformaldehyde. After staining with anti-Japanese flounder IgM mAb and PE-conjugated second antibody as described above, the cells were then subjected to FACScan analysis.

2.5. Sequence analysis

The mRNA sequence (GenBank accession no. XM_020091019.1) and amino acid sequence (GenBank accession no. XP_008321463.1) of *PoCD22* was analyzed using the BLAST program at the National Center for Biotechnology Information (NCBI, http://blast.ncbi.nlm.nih.gov/Blast.cgi). Domain search was performed with the conserved domain search program of NCBI. The theoretical molecular mass and isoelectric point were predicted by using online analysis tool ExPASy (https://web.expasy.org/compute_pi/). Sequence alignment was created with DNAMAN. Phylogenetic analysis was performed with the neighbor-joining algorithm of MEGA 6.0.

2.6. Purification of recombinant protein and preparation of antibody

To construct pETPoCD22, which expresses the extracellular region of PoCD22 (residues 52 to 899), the coding sequence of this region was amplified by PCR with primers F1 (5′-GATATCGGAGATTGGAGCGTG ACCTTT-3′, underlined sequence, *Eco*RV site) and R1 (5′-GATATCCGT CTGGCTGCCGTGG-3′, underlined sequence, *Eco*RV site) and cDNA of spleen was used as the template; the PCR products were ligated with the TA cloning vector T-Simple (TransGen Biotech, Beijing, China), and the recombinant plasmid was digested with *Eco*RV to retrieve the PoCD22-containing fragment, which was inserted into pET32a (Novagen, San Diego, USA) at the *Eco*RV site. rPoCD22 as a fusion protein with the TRX derived from pET32a was purified as described previously [23]. Briefly, *Escherichia coli* BL21 (DE3) (TransGen Biotech, Beijing, China) was transformed separately with pETPoCD22 and pET32a (Novagen, San Diego, USA), which expressing TRX; the transformants were cultured in LB medium at 37 °C to mid-logarithmic phase, and isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the culture to a final concentration of 0.1 mM. After growing at 16 °C for an additional 16 h, the cells were harvested by centrifugation, and His-tagged recombinant PoCD22 (rPoCD22) and rTRX was purified using NiNTA Agarose (QIAGEN, Valencia, USA) following the manufacturer's instruction. The rPoCD22 and rTRX proteins were dialyzed against 1 × PBS (pH 8.0) and then concentrated with PEG20000 (Sigma -Aldrich, St. Louis, USA). The concentrated protein was analyzed in 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie Brilliant Blue R-250 (Fig. S2). The concentration of the purified protein was determined using the Bradford method with bovine serum albumin (BSA) as a standard. Rat anti-rPoCD22 and anti-rTRX antiserum were generated as reported previously [24]. The specificity of the antiserum was confirmed by Western blot analysis as reported previously [25].