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Development of a CD3ε mAb that can identify and *in vitro* activate T cells in large yellow croaker (*Larimichthys crocea*)

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ABSTRACT

As a hallmark molecule of T cells, CD3ε forms a complex with T-cell receptor (TCR) to transduce antigen signals and drive T cell activation, playing a pivotal role in T cell-mediated immune response. However, the lack of specific monoclonal antibodies (mAbs) targeting CD3ε in fish has substantially impeded the study on adaptive immunity in these species. In this study, we constructed NIH/3T3 cells expressing CD3ε of the large yellow croaker (*Larimichthys crocea*) via retroviral transduction. Using these cells as immunogen to immune mice, we generated a mAb that specifically recognized a population of spleen leukocytes after cell fusion and screening. This identified population specifically expressed CD3ε, CD4-1 or CD8α, suggesting that it was T cell. Moreover, immunofluorescence demonstrated that the mAb could bind to the surface of some leukocytes, and it was detected as the IgG1 type. These results confirmed the specificity of this CD3ε mAb and its applicability to identify T cells in the large yellow croaker. Subsequently, we revealed the widespread distribution of CD3ε$^+$ T cells in immune-related tissues including spleen, liver, head kidney, gill and peripheral blood by using this mAb. Upon PHA stimulation, the phosphorylation of mTORC1 and MAPK/ERK were enhanced in CD3ε$^+$ T cells. More importantly, this CD3ε mAb could mimic antigenic signaling to induce T cell activation *in vitro*, since its treatment activated the mTORC1, MAPK/ERK and Ca^{2+} pathways which were crucial for T cell activation. Therefore, we generated a CD3ε mAb, which could not only identify but also *in vitro* activate T cells of the large yellow croaker, providing critical tools for investigating T-cell immune in teleost.

1. Introduction

T lymphocytes originate from hematopoietic stem cells in the bone marrow and undergo maturation in the thymus [1]. Following maturation, these cells circulate through the blood-lymphatic system to peripheral tissues, establishing immune surveillance networks [2]. Functionally specialized T cells are characterized by surface-expressed cluster of differentiation (CD) markers, including CD3, CD4, and CD8, which govern their activation and regulatory mechanisms [3]. Among these T cell surface markers, CD3 is closely associated with T cell activation. CD3 is composed of heterodimeric subunits formed by the γ, δ, ε and ζ chains, including CD3γε, CD3δε and CD3ζζ [4]. Additionally, the T-cell receptor (TCR), which predominantly consists of two peptide chains, α and β (forming the TCRαβ heterodimer), is also central to T cell activation [5]. Typically, TCRαβ heterodimer interacts non-covalently with CD3ε molecules to form the TCR-CD3 complexes, which are expressed on the surface of T cells. However, it is well-established that TCR do not directly recognize soluble antigens [6]. Instead, their recognition is restricted to antigens presented by Major Histocompatibility Complex (MHC) molecules [7]. These MHC-bound antigens are

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processed by antigen-presenting cells (APCs) and displayed on their surface [8]. The binding of MHC-presented antigens to the TCR-CD3 complexes triggers the phosphorylation of an immunoreceptor tyrosine-based activation motif (ITAM) in the cytoplasmic structural domain of the CD3ε-dependent molecule. This process, known as the first signal (antigen signal) transmission, is crucial for the initial activation of T cells and leads to the differentiation and proliferation of effector T cells [9]. Effector T cells secrete cytokines that either directly destroy target cells or enhance the cytotoxic functions of other immune cells, a process known as cellular immunity. T-cell-mediated cellular immunity, along with B-cell-mediated humoral immunity, together constitute adaptive immunity [10].

In mammals, the TCR-CD3 complexes are expressed on all T cells and are essential for their activation. The exposed area of the extracellular domain of CD3ε in TCR-CD3 complexes is higher than that of other subunits and is more easily bound by antibodies [11]. Moreover, compared with other chains, CD3ε is stably expressed in all T cells [12]. Consequently, monoclonal antibodies (mAbs) targeting CD3ε are frequently developed, and cells bound by these antibodies are identified as T cells [13]. CD3ε mAbs are also widely utilized to specifically activate T cells, providing a useful tool for studying T cell functions and immune responses [14,15]. While CD3ε mAbs theoretically offer similar utility in fish immunology, substantial structural divergence between piscine and mammalian CD3ε molecules precludes cross-species antibody application [16]. This evolutionary disparity necessitates species-specific mAbs development for meaningful immunological research in teleost. In fact, several researchers have already initiated work in this area [17]. Bromage et al. developed a mAb against *Oncorhynchus mykiss* CD3ε molecule, and used this mAb to comparatively study the changes in the percentage of *Oncorhynchus mykiss* CD3ε$^+$ T cells upon stimulation with PHA and Con A *in vitro* [18]. Jung et al. developed a mAb against *Paralichthys olivaceus* CD3ε molecule. And during a viral hemorrhagic septicemia virus (VHSV) infection in *Paralichthys olivaceus*, they used this mAb to detect changes in the percentage of CD3ε$^+$ T cells in spleen, liver and trunk-kidney [19]. These case studies validate the feasibility and functional value of species-adapted CD3ε mAbs while highlighting the technical imperative for customized reagent development in non-mammalian models.

Recent studies have increasingly demonstrated that CD3ε$^+$ T cells play a crucial role in the immune response of fish. Fish CD3ε$^+$ T cells were capable of recognizing and eliminating invading pathogens, and they collaborated with B cells to contribute to antibody production [20]. What's more, CD3ε$^+$ T cells in the spleen, head kidney and peripheral blood of *Paralichthys olivaceus* rapidly increased following formalin-inactivated *Edwardsiella tarda* infection [21]. These findings underscore the importance of CD3ε$^+$ T cells in the anti-infection immunity of teleost. After infection with *Flavobacterium columnare*, CD3ε$^+$ T cells in the gill filaments and intestinal mucosa of *Ctenopharyngodon idella* secreted IL-4/13, which coordinated Th2 cell-mediated immune responses, highlighting the critical role of CD3ε$^+$ T cells in mucosal immunity [22]. Furthermore, fish CD3ε$^+$ T cells also participate in maintaining immune tolerance and balancing immune responses [23]. Therefore, these collective findings underscore the necessity of employing CD3ε mAbs to dissect the nuanced contributions of T-cell subsets in teleost.

T cell activation is a precisely regulated process orchestrated by multiple signaling pathways. Upon antigen recognition by the TCR, a cascade of core signaling pathways including Ca^{2+}-NFAT, MAPK/ERK, NF-κB and mTORC1 is triggered. These pathways collectively promote the expression of key effector cytokines such as Interleukin-2 and Interferon-γ, thereby precisely controlling T cell activation amplitude, clonal expansion, and functional differentiation [24]. Our previous work has demonstrated that the MAPK/ERK, mTORC1 and NF-κB pathways cooperatively regulate T cell activation in tilapia [20]. This may indicate that these classical signaling pathways are also tightly correlated with T cell activation in teleost.

To address critical issues in teleost immunology, we successfully developed a species-specific mAb against CD3ε in the large yellow croaker (*Larimichthys crocea*). This commercially valuable marine teleost dominates fisheries along China's continental shelf, particularly in the Yellow Sea, East China Sea, and South China Sea [25]. Known for its distinctive golden hue, the large yellow croaker holds significant economic value along China's eastern coast. Similar to other forms of aquaculture, large yellow croaker farming faces various pathogenic challenges. The development of mAbs against CD3ε in this species addresses the difficulty of localizing T cells in fish immunology research. This advancement enriches the fundamental understanding of aquatic immunology, offers practical guidance for improving aquaculture practices, and enhances the economic value of large yellow croaker farming. Moreover, it helps increase our knowledge of teleost's T cell immunity and provides further evidence to support the study of adaptive immunity in these species.

2. Materials and methods

2.1. Sequence, structure and phylogenetic analysis

The cDNA and amino acid sequences of relevant genes in large yellow croaker were acquired from the National Center for Biotechnology Information (NCBI; https://www.ncbi.nlm.nih.gov). Multiple sequence alignments of CD3ε were carried out using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) and illustrated with The Sequence Manipulation Suite (http://www.bio-soft.net/sms/index.html). The phylogenetic tree was generated via the neighbor-joining (NJ) method in MEGA v.7.0, with 500 bootstrap replicates. The conserved domains were predicted using the Simple Modular Architecture Research Tool (SMART, http://smart.embl-heidelberg.de/), and domain organization was displayed using DOG v.2.0. Protein tertiary structures were predicted with SWISS-MODEL (https://swissmodel.expasy.org/interactive) and visualized in PyMOL v.2.5. Detailed information on the sequences analyzed was provided in Table S1.

2.2. Experimental animals and ethics statement

Healthy large yellow croaker (0.5 kg ± 0.05 kg) were purchased from a mariculture farm (Ningbo, China). All fishes displaying vigorous and healthy characteristics were randomly chosen for the study. BALB/c mice were sourced from the Minhang Laboratory Animal Center at East China Normal University. The experimental protocols followed to the Guidelines for the Care and Use of Experimental Animals established by the Ministry of Science and Technology of China and were sanctioned by the East China Normal University Experimental Animal Ethics Committee. All possible measures were taken to reduce the pain experienced by the experimental animals.

2.3. Retrovirus packaging and cell infection

The full-length coding region of *Lc-CD3ε* was amplified from large yellow croaker cDNA and cloned into MIGR1 vector. BOSC23 and NIH/3T3 cells were cultured at 37 °C, 5 % CO_2 in DMEM (Gibco) containing 10 % fetal bovine serum (FBS, Gibco) and 1 % penicillin/streptomycin (BBI Life Sciences). The MIGR1-*LcCD3ε* plasmid and a pCL-Eco plasmid were co-transfected into BOSC23 cells. Before transfection, 2×10^6 BOSC23 cells were seeded in a 6 cm dish and cultured overnight. Then, the medium was replaced with fresh DMEM containing 1:1000 chloroquine (Sigma). The transfection system contained 10 μg MIGR1-*LcCD3ε* plasmid, 5 μg pCL-Eco plasmid, 50 μL 2.5 M $CaCl_2$ and 500 μL 2 × HEPES. After 8 h, the medium was replaced with fresh DMEM. The supernatant containing retrovirus was harvested after 48 h.

Before cell infection, 6×10^5 NIH/3T3 cells were seeded in a 10 cm dish and cultured overnight. The cells were infected with 600 μL retrovirus in the presence of 5 μg/mL polybrene (Sigma). After 6 h, the

reaction was terminated by adding 3 mL fresh DMEM. The next day the medium was replaced with fresh polybrene-free DMEM. After 48 h, the cells were harvested and used for mouse immunization.

2.4. Preparation of Lc-CD3ε mAb

Healthy female BALB/c mice (6 weeks old) were immunized using NIH/3T3 cells that expressed Lc-CD3ε. The mice received four intraperitoneal (*i.p.*) injections, with a two-week interval between the first and second immunizations, and a one-week interval between subsequent immunizations. The SP2/0 cells were cultured at 37 °C, 5 % CO_2 in 1640 medium (Gibco) containing 10 % FBS. Following the immunization schedule, the BALB/c mice were euthanized, and their spleen cells were fused with SP2/0 cells using polyethylene glycol 4000 (PEG 4000, Sigma). The 96-well plates were used to culture the fused cells along with mouse thymocytes. After more than 10 days of growth in a 1640 medium containing 1 % hypoxanthine, aminopterin, and thymidine (HAT, Gibco) and 10 % FBS, supernatants from hybridoma cells were collected and analyzed by flow cytometry to identify positive clones. The limiting dilution method was used to further sub-clone positive hybridomas. The obtained subclone cells were cultured for 5 days after which the positive cell lines therein were detected using the same method as described above and expanded.

2.5. Lc-CD3ε mAb purification

Lc-CD3ε mAb was purified from mouse ascites using protein G agarose (Invitrogen) according to manufacturer's instructions. Briefly, ascites fluid was incubated with Protein G agarose overnight at 4 °C. Unbound proteins were removed by washing with pre-cooled PBS (pH 7.4), and bound antibodies were eluted with 0.1 M glycine-HCl (pH 2.8). The eluate was immediately neutralized with 1 M Tris-HCl (pH 8.5). After dialysis in PBS, purified Lc-CD3ε mAb was subjected to SDS-PAGE and stained with Coomassie blue.

2.6. Lc-CD3ε mAb isotyping

Subtypes of generated mAbs were classified using the mouse monoclonal antibody isotyping kit (Biodragon). Hybridoma supernatant was added to the ELISA plate and incubated at 37 °C for 30 min. The plates were washed five times with PBS-T (PBS with 0.05 % Tween-20) and then incubated with the HRP-conjugated goat anti-mouse IgG1, IgG2a, IgG2b, IgG3, IgM or IgA at 37 °C for 30 min. After another five washes, TMB substrate was added and incubated at 37 °C for 10 min. Wells showing blue color (positive) indicated the mAbs subtype based on the specific HRP-conjugated antibody used. Eventually, the reaction was stopped by terminating fluid, and the absorbance was measured at 450 nm using a microplate reader.

2.7. Leukocyte isolation

Percoll (GE Healthcare) density gradient centrifugation was used to get leukocytes. 0.33 g sodium heparin (Sangon) and 50 mL of water were used to configure the sodium heparin solution, and the solution was filtered to remove bacteria and set aside. 51 % and 34 % Percoll solutions were configured using 1 × PBS, Percoll and sodium heparin solution (1:100). 4 mL of 51 % Percoll was added to the lower end of a sterile 15 mL centrifuge tube, and 4 mL of 34 % Percoll was added above the 51 % Percoll. Peripheral blood was drawn from the caudal vein of large yellow croaker and quickly mixed with 1 mL of anticoagulant (15 mM sodium citrate, 450 mM NaCl, 0.1 M glucose, 10 mM EDTA, pH 7.0). And it was added into a centrifuge tube and centrifuged at 1200 g, 4 °C for 3 min. After discarding the supernatant, peripheral blood cells were resuspended in Leibovitz's L-15 medium (Gibco). Spleen, head kidney and liver and gill tissues from large yellow croaker were taken and individually ground through a cell mesh sieve to prepare single-cell suspensions, which were diluted with 1 × PBS containing anticoagulant and set aside. The cell suspensions were spread on the upper layer of the centrifuge tube spiked with Percoll solution, and centrifuged at 650 g, 4 °C for 30 min. The leukocytes were located in the middle of 34 % and 51 % of Percoll solution, and the large yellow croaker spleen, head kidney, liver, gill and peripheral blood leukocytes were obtained by centrifuging with 1 × PBS at 500 g, 4 °C for 5 min.

2.8. Leukocyte stimulation

Prior to conducting the phosphorylated protein assay experiments, spleen leukocytes were cultured with Dulbecco's PBS (BBI Life Sciences) at 28 °C for 30 min. Then, 3×10^6 spleen leukocytes were cultured with DMEM containing 10 % FBS and 1 % penicillin/streptomycin in a 24-wells plate at 28 °C. The leukocytes were stimulated by Lc-CD3ε mAb (2 μg/mL), and collected at 5, 15, 45 min for assay. And unstimulated cells were used as control. And for T cell activation, 3×10^6 spleen leukocytes of large yellow croaker were stimulated with PHA (Sigma, 2 μg/mL) for 12 h. Then, the cells were collected for flow cytometry assay.

2.9. Flow cytometry and cell sorting

For screening of positive hybridomas or detection of tissue distribution, 1×10^6 large yellow croaker spleen, head kidney, liver, gill or peripheral blood leukocytes were incubated with hybridoma supernatant or culture supernatant of SP2/0 cells (negative control) on ice for 30 min. The cells were rinsed with FACS buffer (PBS containing 2 % FBS) and then stained with Alexa Fluor 647-conjugated goat anti-mouse IgG H&L (Abcam, 1:2000). After each staining, FACS buffers were used to wash the cells twice. For p-S6 staining, cells were stained with Lc-CD3ε mAb as above, followed by fixation with the fixation/permeabilization solution (BD Biosciences) on ice for 30 min. These cells were then washed twice using the Perm/Wash solution (BD Biosciences) and further stained with PE-conjugated anti-p-S6 Ser240/244 (Biolegend, 1:400). For p-ERK1/2 staining, first stained with FITC-labeled Lc-CD3ε mAb (1 mg/mL, 1:400) and then fixed with Foxp3 Staining Buffer Set (eBioscience) on ice for 2 h, followed by staining with APC-conjugated anti-p-ERK1/2 Thr202/Thy204 (Biolegend, 1:400) on ice for 30 min. All samples were subjected to analysis by flow cytometry (Beckman Colter CytoFLEX), then FlowJo v.10.8.1 software was used to analyze the data. To sort $CD3\varepsilon^+$ and $CD3\varepsilon^-$ T cells, spleen leukocytes were stained with CD3ε hybridoma supernatant as above. The medium was used to resuspend the cells, and then a BD FACS Aria II flow cytometer was utilized to sort them.

2.10. Semi-quantitative assay

$CD3\varepsilon^+$ and $CD3\varepsilon^-$ spleen T cells were sorted out. Subsequently, total RNA was extracted and reverse-transcribed into cDNA. And PCR reaction was used to amplify gene fragments of *β-actin*, *CD3*, *CD4-1*, *CD8α* and *IgM* of large yellow croaker. And the products were added to a 2 % agarose gel for electrophoresis, in order to detect the expression of each gene. All primer sequences were listed in Table S2.

2.11. Immunofluorescence

The large yellow croaker spleen leukocytes were spun onto the slide, and fixed with methanol for 5 min. The leukocytes were incubated with 100 μL 1 % BSA (BBI Life Sciences) for 1 h. The cells were washed twice with PBS-T and once with PBS, each wash lasting 5 min. Next, the leukocytes were incubated with Lc-CD3ε mAb at 37 °C for 1 h and stained with Alexa Fluor 594-conjugated goat anti-mouse IgG H&L (Abcam, 1:800 dilution) for 1 h after washing as above. Finally, the leukocytes were mounted with 2 μL Hoechst 33342 dye (Beyotime) after washing as above, and the images were obtained by fluorescence microscope.

2.12. Western blot

The spleen leukocytes were lysed on ice for 30 min in NP40 lysis buffer (1 % NP40, 150 mM NaCl, 50 mM Tris; pH 7.4) containing 1 % protease inhibitor (MedChemExpress) and 1 % phosphorylase inhibitor (MedChemExpress). The mixture was centrifuged at 13,000 rpm, 4 °C for 10 min. And the supernatant was acquired and separated by 12 % dodecyl sulfate, SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred to nitrocellulose membrane. The nitrocellulose membrane was blocked with 4 % skim milk powder at room temperature for 1 h, and incubated with 1:1000 diluted antibodies for p-mTOR (Ser2448), p-ERK1/2 (Thr202/Tyr204), p-S6 (Ser240/244), p-4EBP1 (Thr37/46), CaM, β-actin from Cell Signaling Technology (CST) at 4 °C overnight, and further incubated with goat anti-rabbit IgG H&L Alexa Fluor 800 (CST, 1:30000) or goat anti-mouse IgG H&L Alexa Fluor 680 (Abcam, 1:10000) at room temperature for 1 h. Images were acquired by Odyssey CLx Image Studio.

3. Result

3.1. Collinearity and protein structure analysis of Lc-CD3ε

In order to search for the *CD3ε* gene in the genome of the large yellow croaker, we conducted a synteny analysis and identified the core component of large yellow croaker *CD3ε* gene on Chromosome VII (Fig. 1A). Its location was significantly different from that in the mouse genome. What's more, only two of the downstream neighboring genes of *Lc-CD3ε* were identical to those in mouse (Fig. 1A). Then we predicted the protein structure of Lc-CD3ε by SMART (Fig. 1B) and compared it with the protein structure of mouse CD3ε. Lc-CD3ε and its mouse homolog shared conserved domains, including IGc2, the transmembrane region and the ITAM motif, but Lc-CD3ε lacked a signal peptide (Fig. 1B). Moreover, comparative tertiary structure analysis demonstrated that Lc-CD3ε had a unique architecture composed of 1 α-helix and 4 β-sheets, contrasting sharply with mouse CD3ε′s configuration of 3 α-helices and 2 β-sheets (Fig. 1C). This marked disparity in structure organization underscored evolutionary divergences in CD3ε molecular architecture between teleost and mammal.

3.2. Phylogenetic analysis of Lc-CD3ε protein

Multiple sequence alignment revealed that despite non-conservation of CD3ε sequences across teleost, reptiles, birds and mammals, they retained conserved IGc2 domains and critical residues, including two cysteine sites (Fig. 2A). Phylogenetic analysis showed that *Larimichthys crocea* and *Collichthys lucidus* CD3ε converged into one lineage (Fig. 2B). Lc-CD3ε and CD3ε of other teleost were located at the roots of phylogenetic trees, forming sister groups with clades of mammals, birds,

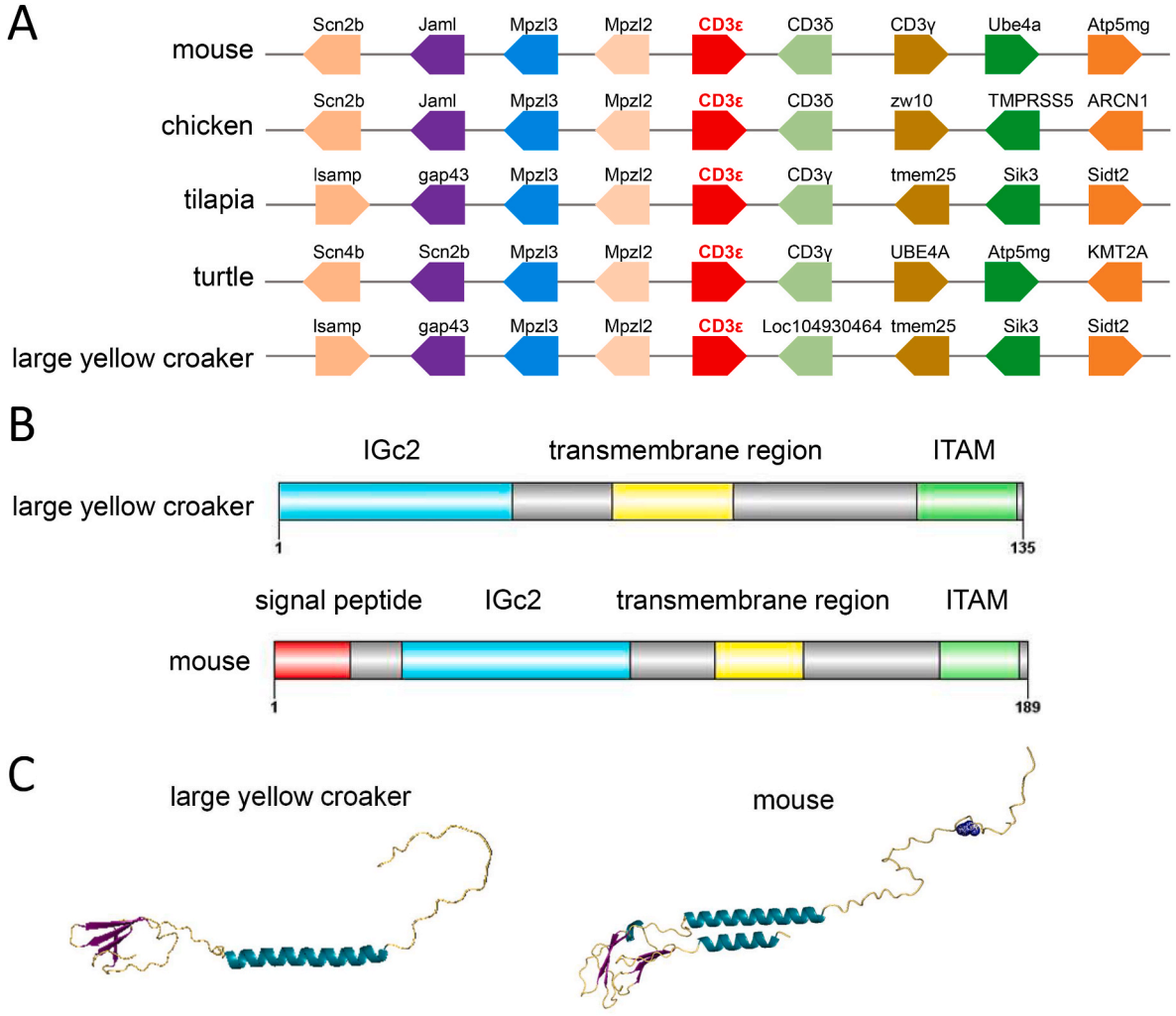

Fig. 1. Collinearity and protein structure analysis of Lc-CD3ε. (A) Synteny and chromosomal location of *CD3ε* gene in vertebrates. (B) Comparison analysis of the protein domain of Lc-CD3ε and Mm-CD3ε. (C) Prediction of protein tertiary structure of Lc-CD3ε and Mm-CD3ε by SWISS-MODEL. Green denotes α-helix, yellow denotes irregular coiling, purple denotes β-sheet.

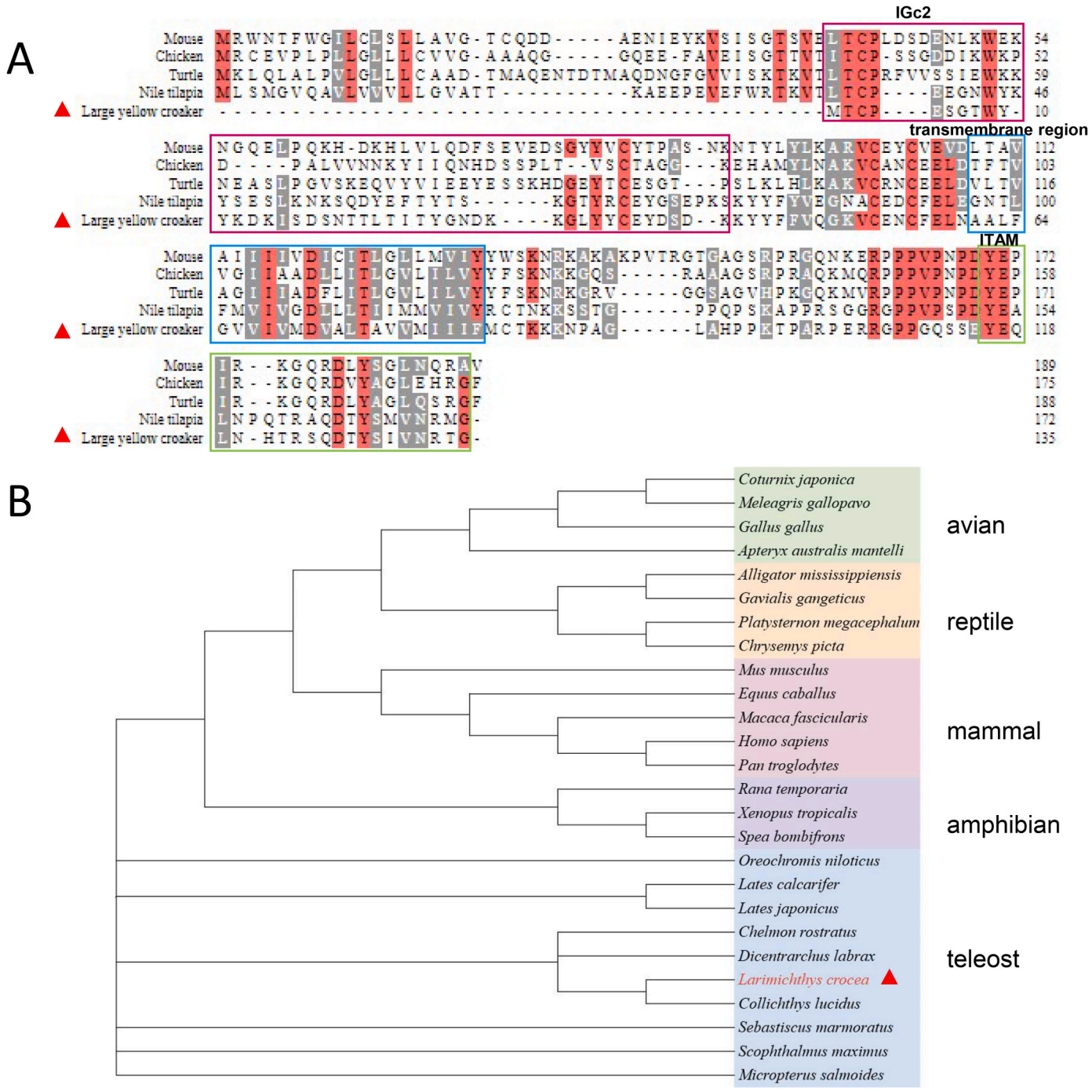

Fig. 2. Phylogenetic analysis of Lc-CD3ε protein. (A) The multiple sequence alignments of Lc-CD3ε protein and homologues from other species. The red amino acid residues are conserved in 80 % of the sequence and the gray amino acid residues are similar. (B) The Phylogenetic tree constructed with the amino acid sequences of CD3ε protein from the indicated species.

reptiles and amphibians (Fig. 2B). These results suggested that the phylogenetic relationship of Lc-CD3ε was consistent with its evolutionary status. In general, the protein sequence of Lc-CD3ε was different from that of mouse CD3ε, and the monoclonal antibodies developed in mice could not be applied to large yellow croaker. Therefore, we needed to develop a monoclonal antibody specifically targeting Lc-CD3ε for further functional investigation.

3.3. Development of Lc-CD3ε mAb

To obtain Lc-CD3ε mAb, we constructed MIGR1-*LcCD3ε* plasmid and transfected plasmid into BOSC23 cells to produce retrovirus (Fig. 3A). Then we collected the supernatant containing retrovirus. After infecting NIH/3T3 cells using the supernatant, cells expressing Lc-CD3ε accounted for 93.8 % of all cells (Fig. 3B), and we also used fluorescence to determine this result (Fig. 3C). Positive hybridoma cells were screened for positive clones (1B10 wells) 10 days after completion of four subcutaneous mice immunizations and fusion of mice spleen cells with SP2/0 cells. And the supernatant produced by this hybridoma cells was able to recognize and conjugate to 33.5 % of the spleen leukocytes (Fig. 3D and E). From the 1B10 hybridoma cells, we screened out a monoclonal hybridoma cell line (1B10F4) and its supernatant was able to recognize

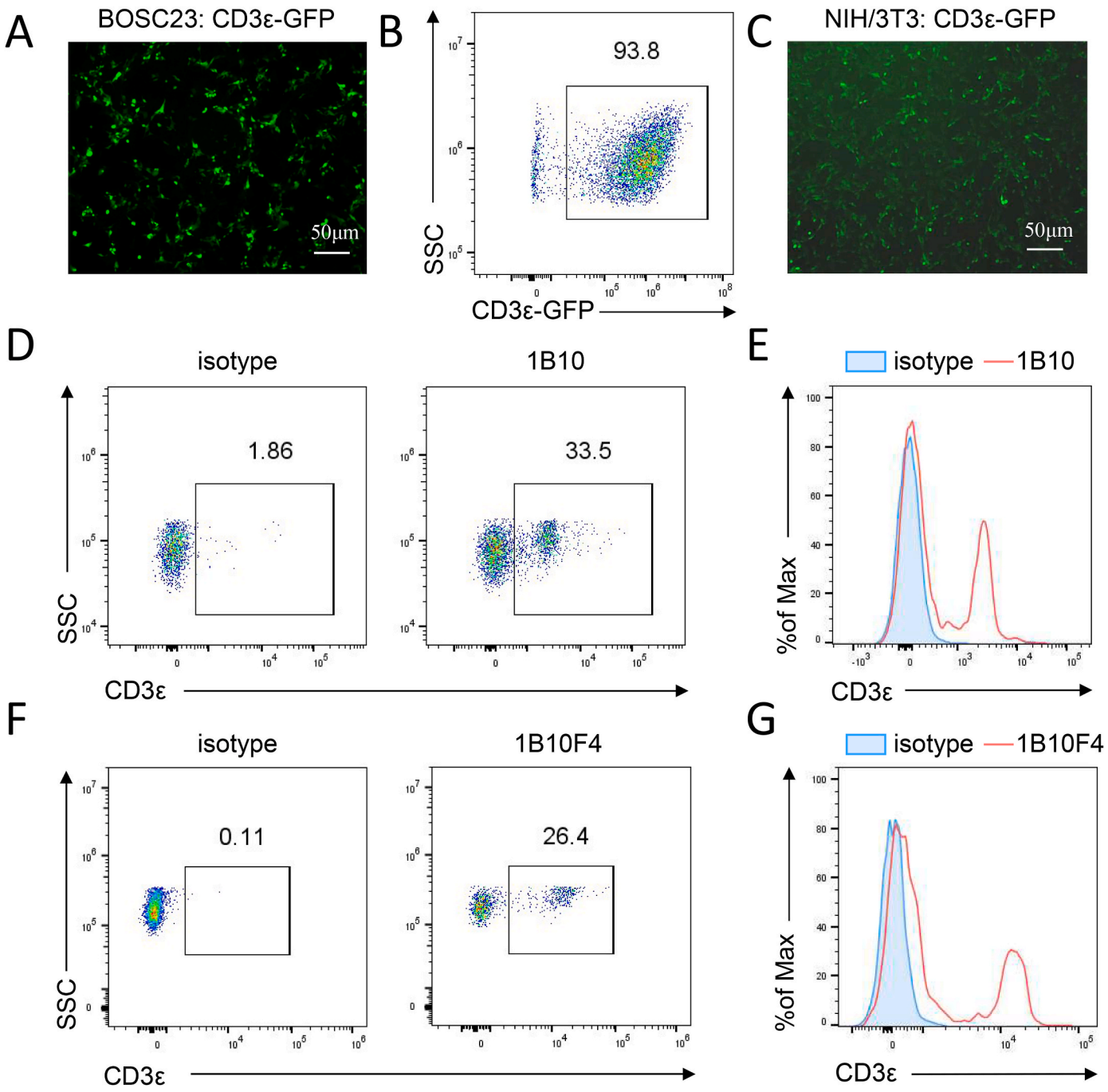

Fig. 3. Development of Lc-CD3ε mAb. (A) BOSC23 cells were transfected with MIGR1-*LcCD3ε* plasmid to produce retrovirus, and the fluorescence microscopy showed the BOSC23 cells expressing Lc-CD3ε. (B, C) Fluorescence microscopy (B) and FACS plots (C) showed the NIH/3T3 cells expressing Lc-CD3ε. (D, E) FACS plots (D) and overlaid histogram (E) showed the large yellow croaker spleen leukocytes were stained with Lc-CD3ε mAb 1B10. (F, G) FACS plots (F) and overlaid histogram (G) showed the large yellow croaker spleen leukocytes were stained with Lc-CD3ε mAb 1B10F4.

and conjugate to 29.6 % of the spleen leukocytes (Fig. 3F and G). Therefore, we selected hybridoma cells that could stably secrete antibodies recognizing certain large yellow croaker spleen leukocytes.

3.4. *Lc-CD3ε mAb specifically recognized CD3ε$^+$ T cells*

To verify the specificity of the antibody, we used this antibody to sort positive and negative cell populations separately to analyze their gene expression profiles. Semi-quantitative analysis indicated that only positive cell population highly expressed molecules specific to T cells, including *CD3*, *CD4-1* and *CD8α* (Fig. 4A). Meanwhile, only the negative cell population expressed the B cell marker molecule *IgM*. And the positive cell population didn't express *Igs*, molecules specific to B cells (Fig. 4A). These findings confirmed that Lc-CD3ε mAb specifically recognized CD3ε$^+$ T cells. In parallel, we purified the Lc-CD3ε mAb from mouse ascites. SDS-PAGE analysis confirmed the obtained mAb exhibited high purity (Fig. 4B). At the same time, the ELISA result showed the Lc-CD3ε mAb we developed was IgG1 subtype antibody (Fig. 4C). In conclusion, we successfully developed a mAb that specifically recognized Lc-CD3ε protein.

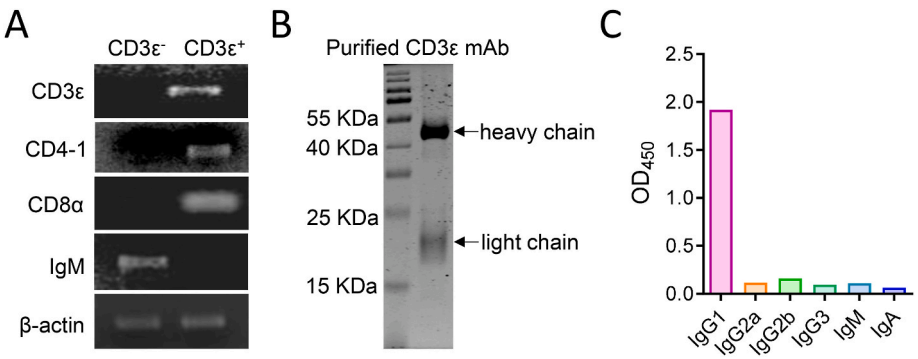

Fig. 4. Lc-CD3ε mAb specifically recognized CD3ε$^+$ T cells. (A) Expression profiles of indicated genes in sorted CD3ε$^+$ and CD3ε$^-$ spleen T cells. (B) Detection of purification of Lc-CD3ε mAb by SDS-PAGE. (C) Detection of subtype of Lc-CD3ε mAb by ELISA.

3.5. CD3ε$^+$ T cells were distributed in various immune tissues

To detect Lc-CD3ε expression, we first performed immunofluorescence tracking of CD3ε$^+$ T cells in spleen leukocytes (Fig. 5A). The results revealed surface expression of Lc-CD3ε on a leukocyte subpopulation. Subsequently, flow cytometric profiling across multiple immune tissues was conducted to establish the tissue-specific expression pattern of T cells. We found that T cells were widespread in immune-related tissues, providing the basis for their rapid response to pathogenic infections (Fig. 5B). Meanwhile, the gills of large yellow croaker contained many T cells, which might suggest their involvement in mucosal immunity (Fig. 5B).

3.6. PHA activated downstream signaling pathways in CD3ε$^+$ T cells

To investigate the activation signals of T cells in large yellow croaker, we used the T cell-specific agonist PHA to induce T cell activation. After 12 h of PHA stimulation, we used flow cytometry to detect the activation levels of the mTORC1 pathway and MAPK/ERK pathway in spleen CD3ε$^+$ T cells of the large yellow croaker. The results showed that the phosphorylation levels of S6 (a key component of the mTORC1 pathway) and ERK1/2 (a key component of the MAPK/ERK pathway), significantly increased after T cell activation, indicating that the mTORC1 pathway and MAPK/ERK pathway are involved in the T cell activation of large yellow croaker (Fig. 6A–B). These evidences further confirmed that large yellow croaker CD3ε$^+$ T cells had activated signaling pathways similar to that of mammals.

3.7. Lc-CD3ε mAb activated downstream signaling pathways in T cells

In mammals, the TCR-CD3 complexes were capable of generating first signals that promoted T cell activation, proliferation and effector functioning thereby enhancing the anti-infection immunity. *In vitro*, we used Lc-CD3ε mAb to mimic the first signal to activate T cells in large yellow croaker. Western blot results showed that phosphorylation levels of ERK1/2 and protein levels of CaM increased after stimulation with Lc-CD3ε mAb and peaked at the 15th min, then down-regulated, but were still higher than normal at the 45th min (Fig. 7). Meanwhile, the phosphorylation levels of key factors of the mTORC1 pathway, including mTOR, S6 and 4EBP1, showed a similar phenomenon after stimulation with Lc-CD3ε mAb (Fig. 7). These evidences suggested that Lc-CD3ε mAb could effectively trigger the activation of T cells and large yellow croaker had a similar T cell activation signaling pathway as mammals. All in all, we developed a CD3ε mAb that could be used to simulate first signals *in vitro* to induce T cell activation.

4. Discussion

Previous studies on T cell research in large yellow croaker have primarily relied on polyclonal antibodies (pAbs) and antibodies targeting non-canonical T cell marker molecules such as Zap70 [26]. Studies in large yellow croaker have localized CD4-2$^+$ T cells using rabbit anti-LcCD4-2/CD154 pAbs, revealing BTLA-HVEM-mediated inhibition of T cell activation [27]. Building upon this foundation, rabbit anti-LcCD40 pAbs were generated, demonstrating CD40/CD154 co-stimulation modulates T cell-mediated immune responses in large yellow croaker [28]. Zap70$^+$ T cells in large yellow croaker were localized using Zap70 antibody, with IL-2 signaling coupled to the MAPK axis shown to promote T cell proliferation and differentiation [29]. pAbs exhibit multi-epitope binding heterogeneity, elevating cross-reactivity risks with structurally homologous proteins. Antibodies targeting non-canonical T cell markers like Zap70 have the potential to cross-react with non-T lymphocyte subsets. Therefore, we need to develop Lc-CD3ε mAb to specifically recognize T cells and study T cell functions.

Before initiating the antibody preparation experiments, we conducted structural and developmental characteristic analyses of Lc-CD3ε. Our findings indicate that although Lc-CD3ε and mammalian CD3ε share similar functional domains and key sites, they exhibit significant differences in structure characteristics and protein three-dimensional structures. These differences indicate that mAbs developed for mammals cannot be directly applied to teleost due to variations in binding sites. In addition, the current preparation of T cell-related mAbs in fish primarily relies on the prokaryotic recombinant system, which cannot restore the natural conformation and immunogenic activity of antigens, significantly limiting antibody development [30]. Currently, T cell-related antibodies have been developed in only a few fish species [31,32]. In this study, we constructed antigen chimeric cells as immunogens and combined flow cytometry with gene expression analysis to develop a technology for screening and identifying monoclonal antibodies. Using this approach, we successfully developed a specific monoclonal antibody targeting Lc-CD3ε, providing a reliable tool for studying the effector functions and regulatory mechanisms of T cells in large yellow croaker.

We compared the distribution of CD3ε$^+$ T cells in lymphoid and non-lymphoid tissues of large yellow croaker using flow cytometry. The results showed that CD3ε$^+$ T cells were widely distributed in the lymphoid tissues of large yellow croaker and the highest proportion was found in the spleen and liver. Experimental results in two morphs of brown trout (*Salmo trutta*) showed that CD3ε$^+$ T cells were also concentrated in the spleen and liver, suggesting that the spleen and the liver were also important peripheral immune organs in fish [33,34]. Studies had shown that a high abundance of CD3ε$^+$ T cells was present in the spleen, head kidney and peripheral blood of Japanese flounder (*Paralichthys olivaceus*). These findings, together with our results, suggested that CD3ε$^+$ T cells played an important role in peripheral immunity in fish [35]. What's more, the study in rainbow trout (*Oncorhynchus mykiss*) found that its skin had a high percentage of CD3ε$^+$ T cells, which was not

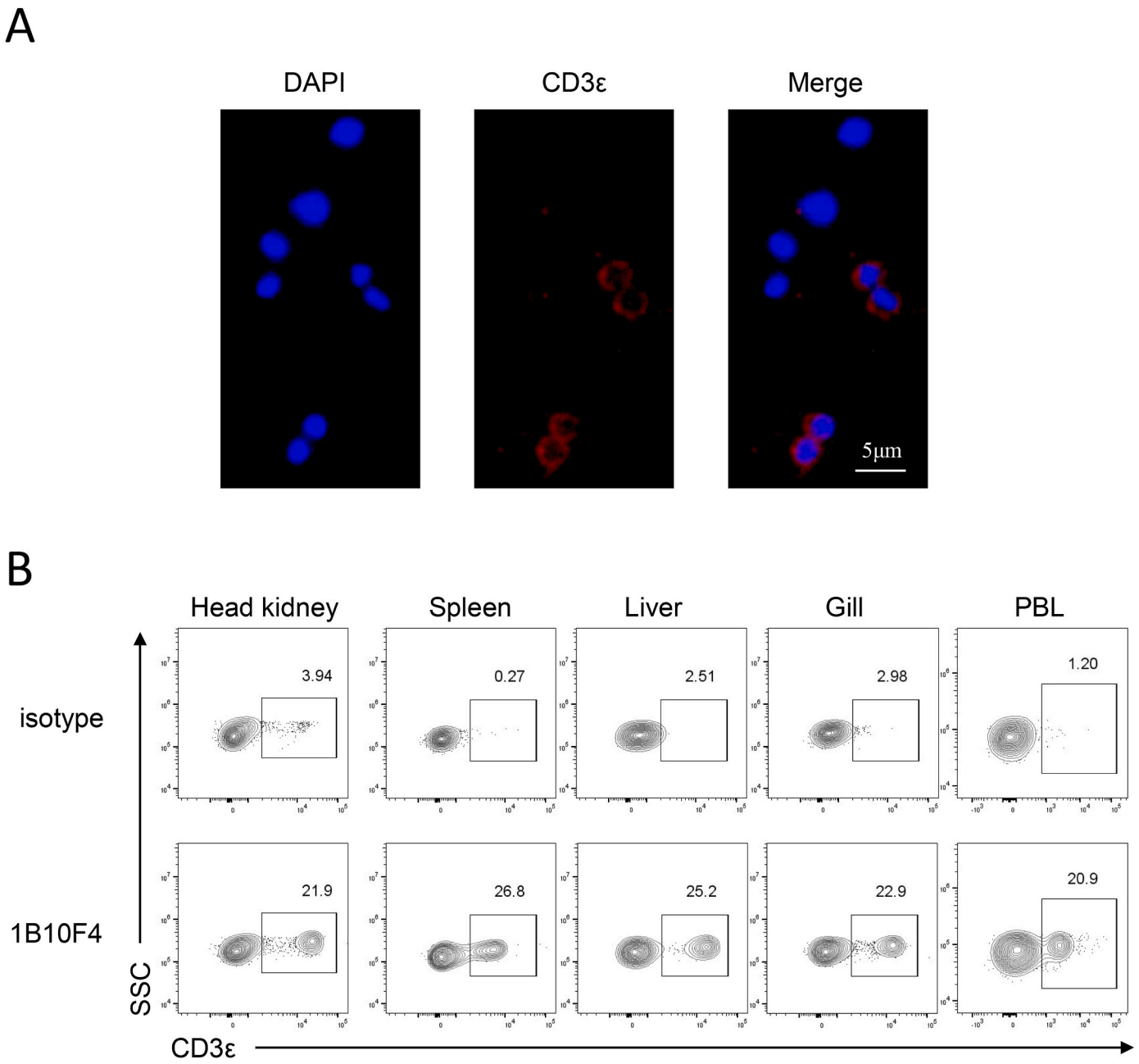

Fig. 5. CD3ε$^+$ T cells were distributed in various immune tissues. (A) Lc-CD3ε protein on T cells' surface was detected by immunofluorescence, Hoechst 33342 dye binds to the nucleus in blue and Lc-CD3ε mAb located on the surface of T cells binds to fluorescent secondary antibody PE in red. (B) CD3ε$^+$ T cells in different tissues including head kidney, spleen, liver, gill and peripheral blood.

consistent with the results we obtained [36]. This might imply that CD3ε$^+$ T cells could play an important function in rainbow trout mucosal immunity and suggest differences in CD3ε$^+$ T cell content in different tissues of fish.

In mammals, T cell activation is regulated by the coordinated control of multiple signaling pathways, including Ca^{2+}-NFAT, MAPK/ERK, and mTORC1 [37–39]. These pathways integrate upstream antigen and co-stimulatory signals to regulate T cell activation, proliferation and effector functions. In our previous studies, we demonstrated the existence of classical signaling pathways for T cell activation in Nile tilapia similar to those in mammals, including Ca^{2+}-NFAT, MAPK/ERK and mTORC1 signaling [40–43]. And these signaling pathways could regulate biological processes such as proliferation, differentiation and apoptosis of T cells in fish. Consistent with our findings, the phosphorylation levels of S6 and ERK1/2 in CD3ε$^+$ T cells were significantly increased after being stimulated with PHA, suggesting that these pathways were involved in the activation of T cells in large yellow croaker. Previous studies have shown that *in vitro* co-stimulation with PHA and rLcIL-2 activated the JAK/Stat 5, mTORC1 and MAPK/ERK pathways downstream of T cells and promoted T cell proliferation [29]. This is highly consistent with our findings, suggesting that large yellow croaker T cells may also have T cell activation signaling pathways similar to those of mammals.

In mammals, anti-CD3ε mAbs can mimic the first signal *in vitro* to induce T cell activation and have become a powerful tool for studying T cell functions and regulatory mechanisms. However, in lower vertebrates, related tools are extremely limited, restricting the understanding of their T cell immunity. In our previous study on tilapia, the developed CD3ε mAb was capable of inducing T cell activation in tilapia [20]. Moreover, we found that the full activation and proliferation of tilapia T

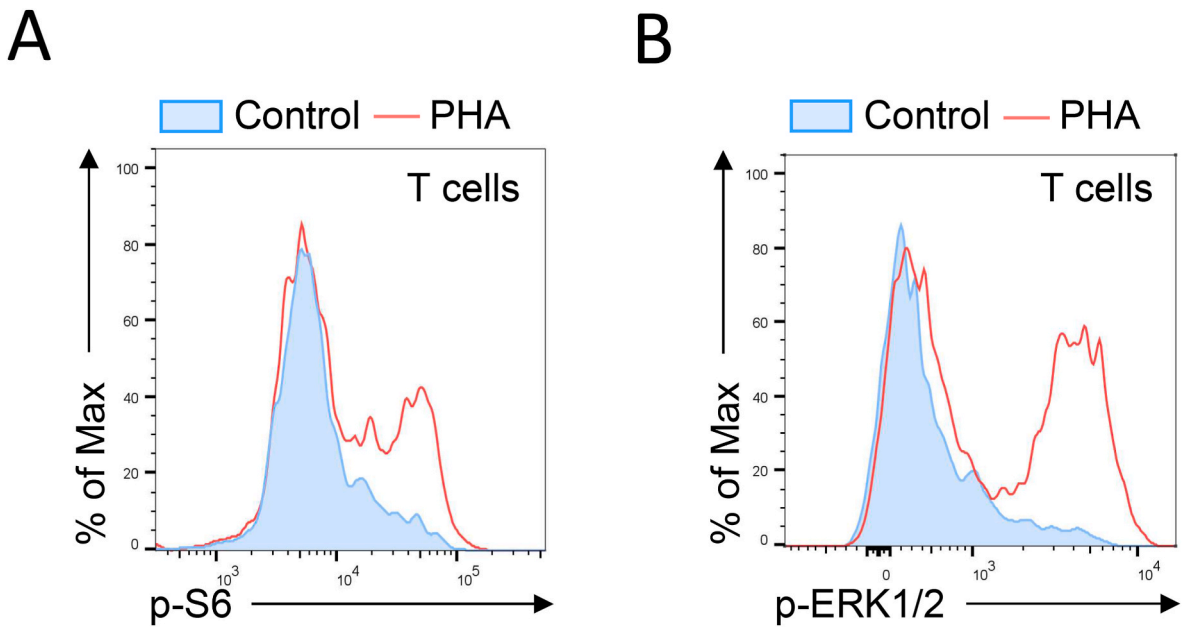

Fig. 6. PHA activated downstream signaling pathways in CD3ε^{+} T cells. (A, B) ERK1/2 and S6 phosphorylation levels in T cells were measured by flow cytometry after 12 h of stimulation with 2 μg/mL PHA.

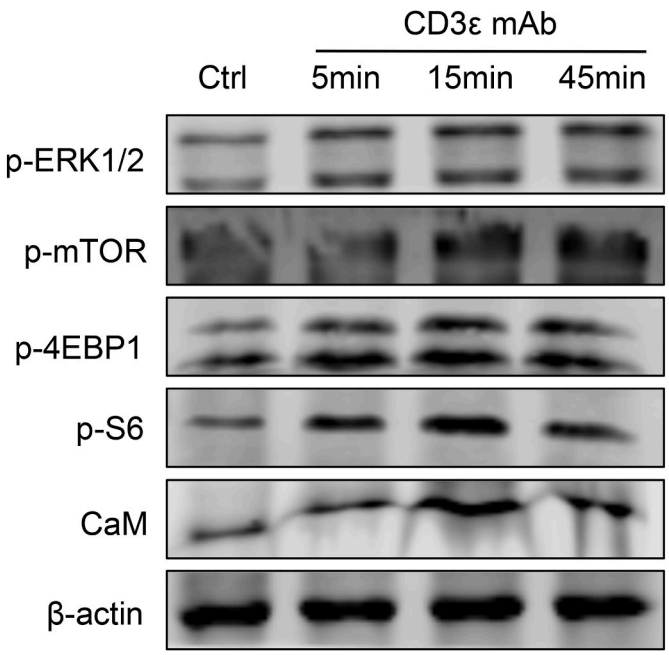

Fig. 7. Lc-CD3ε mAb activated downstream signaling pathways in T cells. (A) Detection of mTOR, ERK1/2, 4EBP1 and S6 phosphorylation levels and CaM protein levels after 5, 15 and 45 min of stimulation by 2 μg/mL Lc-CD3ε mAb.

cells required not only the first signal but also a co-stimulatory signal mediated by CD28 [20,44–46]. These findings suggested that CD3ε mAbs can effectively activate teleost's T cells *in vitro*, providing a valuable tool for studying the initiation of adaptive immunity, immune cell signaling pathways and downstream coupling mechanisms in teleost. In this study, the developed Lc-CD3ε mAb induced the activation of T cell activation-related pathways, mTORC1 and MAPK/ERK, confirming that this antibody can also serve as a tool to mimic the first signal of T cells in large yellow croaker. Future studies may focus on developing a Lc-CD28 mAb to determine whether combined stimulation with Lc-CD3ε and Lc-CD28 mAbs can fully activate T cells in large yellow croaker. This will help verify whether this combined activation is a general mechanism in teleost.

In conclusion, we developed a mAb against Lc-CD3ε in large yellow croaker. Using this mAb, we found that CD3ε^{+} T cells are widely distributed in immune-related tissues, providing molecular potential for their active response to pathogen infection. Furthermore, we discovered that the mTORC1 and MAPK/ERK pathways are involved in the activation of CD3ε^{+} T cells in large yellow croaker. Additionally, using this antibody to mimic first signals *in vitro* induced the activation of T cell activation-related pathways, establishing an *in vitro* T cell activation model. Our study provides a valuable tool for exploring T cell-mediated anti-infection immunity in large yellow croaker.

CRediT authorship contribution statement

Zhichao Fang: performed experiments, Formal analysis, and wrote the paper. **Yi Cao:** performed experiments. **Haokai Chen:** performed experiments. **Jie Cheng:** performed experiments. **Ming Geng:** performed experiments. **Jiong Chen:** performed experiments. **Yinnan Mu:** performed experiments. **Kang Li:** performed experiments, conceived the project, designed experiments and wrote the paper. **Jialong Yang:** conceived the project, designed experiments and wrote the paper. **Xiumei Wei:** conceived the project, designed experiments and wrote the paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2025.110461.

Data availability

No data was used for the research described in the article.

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