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Full T-cell activation and function in teleosts require collaboration of first and co-stimulatory signals

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ABSTRACT

Mammalian T-cell responses require synergism between the first signal and co-stimulatory signal. However, whether and how dual signaling regulates the T-cell response in early vertebrates remains unknown. In the present study, we discovered that the Nile tilapia (*Oreochromis niloticus*) encodes key components of the LAT signalosome, namely, LAT, ITK, GRB2, VAV1, SLP-76, GADS, and PLC-γ1. These components are evolutionarily conserved, and CD3ε mAb-induced T-cell activation markedly increased their expression. Additionally, at least ITK, GRB2, and VAV1 were found to interact with LAT for signalosome formation. Downstream of the first signal, the NF-κB, MAPK/ERK, and PI3K-AKT pathways were activated upon CD3ε mAb stimulation. Furthermore, treatment of lymphocytes with CD28 mAbs triggered the AKT-mTORC1 pathway downstream of the co-stimulatory signal. Combined CD3ε and CD28 mAb stimulation enhanced ERK1/2 and S6 phosphorylation and elevated NFAT1, c-Fos, IL-2, CD122, and CD44 expression, thereby signifying T-cell activation. Moreover, rather than relying on the first or co-stimulatory signal alone, both signals were required for T-cell proliferation. Full T-cell activation was accompanied by marked apoptosis and cytotoxic responses. These findings suggest that tilapia relies on dual signaling to maintain an optimal T-cell response, providing a novel perspective for understanding the evolution of the adaptive immune system.

Keywords: *Oreochromis niloticus*; CD3; CD28; T cells; Adaptive immunity; Evolution

INTRODUCTION

The emergence of the T-cell-mediated adaptive immune system in fish played a critical role in facilitating the rapid occupation of a favorable ecological niche by jawed vertebrates (Anderson et al., 2004). Recently, many studies have highlighted the importance of T-cell-mediated adaptive immunity in teleosts (Ai et al., 2022; Jung et al., 2021; Mu et al., 2022; Tafalla et al., 2016). Once T cells recognize the major histocompatibility complex (MHC)-antigenic peptide complex on the surface of antigen-presenting cells (APCs), they rapidly activate, proliferate, differentiate, and release cytokines to resist pathogenic bacterial invasion (Ashfaq et al., 2019). In recent years, our understanding of the immune system in certain fish species has seen significant improvements. However, the scarcity of adequate tools to study the fish immune response continues to impede research on the mechanisms and evolution of the fish immune system.

Appropriate T-cell activation is essential to initiate an appropriate immune response. Mammalian T-cell activation, proliferation, and differentiation require two critical stimulatory signals: the interaction of the T-cell receptor (TCR)/CD3 complex with MHC on APCs and the binding of the co-stimulatory molecule CD28 to the B7 family ligands on APCs (Cheng et al., 2011; Liu et al., 2020b; Mariuzza et al., 2020; Sanchez-Lockhart et al., 2011). Dependent on conformational changes, the immunoreceptor tyrosine-based activation motif (ITAM) in the ζ chain of the TCR/CD3 complex is phosphorylated by the leukocyte C-terminal Src kinase (LCK), which further initiates the recruitment and activation of zeta chain of T-cell receptor-associated protein kinase 70 kDa (ZAP-70) (Au-Yeung et al., 2018). Subsequent phosphorylation of the SH2-domain-containing leukocyte protein 76 kDa (SLP-76) by ZAP-70 is indispensable for

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interleukin 2 (IL-2) inducible T-cell kinase (ITK) activation, which is then recruited to the plasma membrane (Sela et al., 2011). Changes in the conformation of ITK facilitate the activation of downstream effectors via formation of the linker for activation of T cells (LAT) signalosome (Schwartzberg et al., 2005). Growth factor receptor-bound protein 2 (GRB2), ITK, SLP-76, GRB2-related adaptor protein 2 (GADS), vav guanine nucleotide exchange factor 1 (VAV1), and phospholipase C gamma 1 (PLC-γ1) are known to interact with LAT in mammals, which is for downstream signaling cascades (Bunnell et al., 2000). PLC-γ1, once activated, hydrolyzes the membrane lipid phosphatidylinositol 4,5-bisphosphate into two second messengers: inositol polyphosphates and diacylglycerols (Smith-Garvin et al., 2009). Subsequently, with the help of second messengers, downstream signaling cascades are activated, including Ca^{2+}-calcineurin-NFAT, NF-κB, MAPK/ERK, and mTOR signaling (Gorentla & Zhong, 2012). These signaling cascades ultimately facilitate T-cell activation, proliferation, and effector function (Gorentla & Zhong, 2012). The first signal facilitates antigen binding to specific co-receptors on the T-cell surface, prompting initial T-cell activation. As a critical co-receptor, CD3 is involved in the assembly, stabilization, and signal transduction of the TCR/CD3 complex. This complex transmits the activation signal, which is generated when the TCR binds to the antigen, into the intracellular compartment, ultimately leading to T-cell activation (Kuhns et al., 2006). Monoclonal antibodies targeting the CD3 ε chain (CD3ε mAbs) have been developed to induce mammalian T-cell activation *in vitro* (Chitnis et al., 2022). The binding of CD3 mAbs strongly initiates T-cell activation and IL-2 receptor expression. Moreover, CD3 mAb stimulation can enhance the production of IL-2, IL-3, and interferon γ (IFN-γ) (Sauerwein et al., 1988).

However, without the second signal, the interaction of the TCR with the MHC-antigenic peptide complex is not sufficient to trigger full T-cell activation, leading to a hypo-responsive state (Caporali et al., 2014). The second signal is a non-specific, co-stimulatory signal generated by the interaction of multiple pairs of co-stimulatory molecules on APCs and corresponding TCRs, e.g., CD28 and CD80/CD86, 4-1BB and 4-1BBL, and CD40 and CD40L (Chen & Flies, 2013). CD28, as the most important co-stimulatory molecule, is characterized by an extracellular variable immunoglobulin-like structural domain and is constitutively expressed in T cells (Beyersdorf et al., 2015; Esensten et al., 2016). The involvement of CD28 enhances full T-cell activation, cell survival, and cytokine secretion (Marinari et al., 2004). Moreover, cross-linking CD28 with CD28 mAbs in the presence of alloantigen, T-cell mitogen (PHA), or CD3 mAbs greatly enhances the IL-2 production, activation, proliferation, and cytotoxicity of T cells (June et al., 1990; Jung et al., 1987; Lesslauer et al., 1986). However, CD28 signaling alone does not induce T-cell proliferation (Hara et al., 1985). Although numerous mammalian studies have elucidated that dual signaling effectively initiates T-cell function (Nguyen et al., 2016; Sloan-Lancaster et al., 1993), its potential regulatory role in T-cell response remains unknown in early vertebrates.

An increasing number of studies have demonstrated that signal transduction downstream of the TCR in teleosts is similar to that in mammals (Rodríguez-Caparrós et al., 2020), but many obstacles remain in identifying the different components and mechanisms that trigger TCR signaling, including the lack of mAbs (Randelli et al., 2008). Recently, various mAbs have been developed against T-cell markers in teleosts, including DLT15, which specifically targets peripheral and thymocyte T cells in European sea bass, and 6D1 and 6C10, which specifically target CD4 and CD8, respectively, in common carp (Abelli et al., 1999; Laing & Hansen, 2011). Moreover, several mAbs have been developed against the first signal (CD3) in teleosts, including rainbow trout, olive flounder, Japanese flounder, and grass carp (Boardman et al., 2012; Jung et al., 2017; Qin et al., 2021; Tang et al., 2017). Furthermore, mAbs have been developed to target the co-stimulatory molecule CD28 in olive flounder (Xing et al., 2021). However, how the first signal and co-stimulatory signal together regulate the T-cell response in teleosts remains poorly understood. In the present study, we revealed conserved components of the LAT signalosome in Nile tilapia (*Oreochromis niloticus*) that may be involved in TCR downstream signal transduction upon CD3ε mAb-induced T-cell activation. Furthermore, we demonstrated the regulatory role of the dual signal in full T-cell activation, proliferation, apoptosis, and cytokine release. To the best of our knowledge, this is the first study to describe the regulatory mechanism of dual signaling-mediated T-cell response in a non-mammalian vertebrate. Collectively, these findings provide a valuable reference for understanding the role of dual signaling-induced TCR signal transduction, as well as the evolution of T-cell-mediated adaptive immunity.

MATERIALS AND METHODS

Ethics statement and experimental animals

Nile tilapias (2 g in weight) were purchased from a fish farm in Guangzhou, Guangdong Province, China, and cultured in 120 L tanks at 28 °C with continuous aeration and daily feeding at the biological station of East China Normal University. Healthy tilapias were used for the experiments once reaching 15 g in weight. All experimental procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the Ministry of Science and Technology of China and were approved by the East China Normal University Experimental Animal Ethics Committee (permit No. AR2021-245).

Sequence, structure, and phylogenetic analysis

The cDNA and amino acid sequences of related genes were obtained from the NCBI GenBank database (https://www.ncbi.nlm.nih.gov/) and were analyzed using the BLAST algorithm (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The protein domains were predicted by SMART (http://smart.embl.de/) and domain organization was displayed using DOG v2.0 software. Multiple sequence alignment of the amino acids was performed using Clustalx v1.83. The protein tertiary structures were predicted using SWISS-MODEL (https://swissmodel.expasy.org/) and displayed with PyMOL software v2.3.2.0. The phylogenetic tree was constructed using MEGA v7.0 with the neighbor-joining algorithm. The accession numbers of selected genes are listed in Supplementary Table S1.

Leukocyte isolation

Spleen and head kidney leukocytes were isolated according to our previous study (Wei et al., 2019). Briefly, Percoll (GE Healthcare, USA) was mixed with 10×phosphate-buffered saline (PBS) at a ratio of 9:1, then diluted to 52% Percoll and 34% Percoll with L-15 medium (Gibco, USA), respectively.

The spleen was harvested, and a single-cell suspension was prepared with L-15 medium. We added 4 mL of 52% Percoll, 34% Percoll, and cell suspension, respectively, into a 15 mL centrifuge tube, which was then centrifuged at 500 ×*g* for 35 min at room temperature with the lowest acceleration and deceleration. Leukocytes between the 52% and 34% Percoll layer were collected, washed with L-15 medium, and resuspended with Dulbecco's modified Eagle medium (DMEM, Sangon Biotech, China) with 10% fetal bovine serum (FBS, Gibco, USA) for future assay. According to our previous study, almost all isolated spleen leukocytes are lymphocytes (Ai et al., 2022; Wei et al., 2019).

Leukocyte stimulation

The spleen leukocytes (3×10^6) were cultured in a 24-well plate with DMEM containing 10% FBS and 1% penicillin/streptomycin (Sangon Biotech, China) at 28 °C. The cells were then stimulated with previously generated CD3ε and CD28 mAbs (2 μg/mL) (Li et al., 2023), and collected at indicated times for assay. Unstimulated leukocytes were used for the control. For the phosphorylation assays, the spleen leukocytes were resuspended in Dulbecco's PBS at 28 °C for 30 min to rest the phosphorylated proteins. After that, the cells were transferred to DMEM (10% FBS) and stimulated with CD3ε or CD28 mAbs at 28 °C. The leukocytes were collected at indicated times for western blotting and immunofluorescence assays.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from the spleen leukocytes using TRIzol reagent (Invitrogen, USA) according to the provided instructions. After treatment with gDNA Purge for 5 min at 42 °C, the RNA was reverse-transcribed using the First-Strand cDNA Synthesis SuperMix (Novoprotein, China). The resulting cDNA was diluted 1:10 to serve as a template, and qRT-PCR was performed using the NovoStart SYBR qPCR SuperMix Plus (Novoprotein, China) on a CFX Connect Real-Time System (BioRad, USA). Relative mRNA expression was calculated using the $2^{-\Delta\Delta CT}$ method, with β-actin used as the reference gene. All primer information is listed in Supplementary Table S2.

Western blot assay

The spleen leukocytes were lysed in NP40 lysis buffer containing 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), protease inhibitor cocktail, and phosphatase inhibitor cocktail III (MedChemExpress, USA) on ice for 30 min. The supernatant was harvested after centrifugation at 10 000 ×*g* and 4 °C for 10 min, then mixed with sodium dodecyl sulfate (SDS)-loading buffer and boiled at 100 °C for 10 min. The sample was separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and subjected to western blot analysis using primary antibodies (1:1 000), including anti-p-IKKα/β (Ser176/180, #2697), anti-IKKα/β (#2694), anti-p-IκBα (Ser32, #2859), anti-IκBα (#4812), anti-p-NF-κB p65 (Ser468, #3039), anti-NF-κB p65 (#9936), anti-p-c-Raf Ser-338 (#9427), anti-c-Raf (#9422), anti-p-MEK1/2 (Ser217/221,#9936), anti-Mek1/2 (#8727), anti-p-p44/42 MAPK (ERK1/2) (Thr202/Tyr204, #4370), anti-p44/42 MAPK (ERK1/2,#9102), anti-p-MEK1/2 (Ser217/221, #8221), anti-MEK1/2 (#8727), anti-p-c-Fos (ser32, #5348), anti-c-Fos (#2250), anti-PI3 Kinase p110α (#4249), anti-p-AKT (Thr308,#13038), anti-p-AKT (Thr473, #4060), anti-AKT (#4697), anti-p-S6 (Ser240/244, #5364), anti-S6 (#2217), anti-p-4EBP1 (Thr37/46, #5123), anti-4EBP1 (#9959), and anti-β-actin (#3700) purchased from Cell Signaling Technology. Primary antibody binding was then detected using secondary antibodies, 1:30 000 diluted goat anti-rabbit IgG H&L Alexa Fluor 800 (#5151, Cell Signaling Technology, USA) or 1:10 000 diluted goat anti-mouse IgG H&L Alexa Fluor 680 (#ab175775, Abcam, United Kingdom), at room temperature for 1 h. After each incubation, the membranes were washed three times using PBS with Tween 20 (PBST). Finally, the blots were scanned using Odyssey CLx Image Studio (USA).

Immunofluorescence assay

The spleen leukocytes were centrifuged onto slides using Cytospin 4 (Thermo Scientific, USA) at 1 000 ×*g* for 3 min, then fixed with methyl alcohol for 5 min. After the slides were blocked with 1% bovine serum albumin (BSA) at 37 °C for 1 h, the samples were washed twice with PBST and once with PBS. The samples were subsequently incubated with 1:200 diluted mouse-anti CD3ε or CD28 mAbs, rabbit anti-p-S6 (Ser240/244), rabbit anti-p-ERK1/2 (Thr202/Tyr 204), or mouse anti-β-actin primary antibodies at 37 °C for 1 h. After washing, the cells were incubated with 1:800 diluted Alexa Fluor 488-conjugated goat anti-mouse IgG H+L (Abcam), Alexa Fluor 594-conjugated goat anti-mouse IgG H+L (Abcam), or Alexa Fluor 594-conjugated goat anti-rabbit IgG H+L (Abcam) secondary antibodies at 37 °C for 1 h. For the CD3 and CD28 co-localization assay, the cells were first stained with mouse anti-CD28 mAbs and Alexa Fluor 594-conjugated goat anti-mouse IgG H+L, followed by incubation with FITC-conjugated mouse anti-CD3ε mAbs at 37 °C for 1 h. After washing three times, 4',6-diamidino-2-phenylindole (DAPI) staining solution (Beyotime, China) was added, with the slides then sealed with a coverslip and observed using a Zeiss ApoTome microscope (Germany).

Co-immunoprecipitation (Co-IP) assay

The coding sequence (CDS) regions with a Flag or HA tag, including LAT-Flag, GRB2-HA, ITK-HA, VAV1-HA, PLC-γ1-HA, GADS-HA, and SLP-76-HA, were amplified from the tilapia cDNA with corresponding primers (Supplementary Table S2), then ligated into pEGFP-C1 plasmid to construct transfection vectors. The HEK293T cells (2×10^6) were seeded into 6 cm^2 dishes overnight, which were co-transfected with 5 μg of the LAT-Flag-pEGFP-C1 plasmid and 5 μg of the partner-HA-pEGFP-C1 plasmid. The cells were collected at 48 h post-transfection, then lysed with NP40 as above. The supernatants were collected and incubated with 10 μL of anti-Flag Ab-conjugated agarose beads (Sigma-Aldrich, USA) at 4 °C overnight with shaking. Subsequently, the beads were washed five times with 1 mL of NP40 lysis buffer, mixed with SDS-loading buffer and heated at 100 °C for 10 min, followed by western blot analysis.

Flow cytometry

The spleen and head kidney leukocytes were stained with mouse anti-tilapia CD28 mAbs (1 : 400) in FACS buffer (2% FBS in PBS) on ice for 30 min, then washed twice with FACS buffer. The cells were subsequently incubated with Alexa Fluor 647-conjugated goat anti-mouse IgG H+L (1 : 2 000; Abcam) on ice for 30 min, then washed twice. After that, the leukocytes were stained with FITC-conjugated CD3ε mAbs (1 : 400) on ice for another 30 min and washed twice. The cells were finally resuspended in FACS buffer and collected

on a BD Canto-II flow cytometer. The data were analyzed using FlowJo software v10.0.7.

Cell proliferation assay

Spleen leukocytes (1×10^6) were labeled with 10 μmol/L carboxyfluorescein succinimidyl ester (CFSE, Invitrogen, USA) according to the manufacturer's instructions. After washing twice with L15 medium, the cells were resuspended in DMEM (10% FBS) and cultured in a 24-well plate with or without 2 μg/mL CD3ε or CD28 mAbs. The cells were harvested at 48 h and stained with CD3ε mAbs as above. Subsequently, 7AAD (1:400; BioLegend, USA) was added before the samples were subjected to flow cytometry assay.

Apoptosis assay

The spleen leukocytes were stimulated with CD3ε and CD28 mAbs for specified durations, as described above. The cells were first stained with CD3ε mAbs, then twice washed with FACS buffer. Subsequently, the cells were stained with APC-conjugated Annexin V antibodies (1 : 400; BioLegend) in Annexin V binding buffer (0.14 mol/L NaCl, 0.01 mol/L HEPES/NaOH, 2.5 mmol/L $CaCl_2$, pH 7.4,) at room temperature for 15 min, with 7AAD (1:400) then added before flow cytometry assay.

Statistical analysis

All results are presented as mean±standard error of the mean (*SEM*). Significant differences were determined using a two-tailed Student *t*-test. *P*-values were indicated as *: $P<0.05$; **: $P<0.01$; ***: $P<0.001$.

RESULTS

Tilapia possesses intact and evolutionarily conserved LAT signalosome components

Downstream of TCR signaling cascades, the LAT signalosome plays an essential role in signal transduction to ensure a proper T-cell response. Here, the central component of the LAT signalosome was found to be located on chromosome LG4 in the Nile tilapia (Figure 1A). Based on synteny analysis, both the location and orientation of LAT and its adjacent genes, including *SH2B1, ATXN2L, SPNS1, NFATC2IP, and SGF29*, were evolutionarily conserved among the teleost species (Figure 1A). In addition to LAT, other components that form the signalosome in mammals, including ITK, GRB2, SLP-76, GADS, VAV1, and PLC-γ1, were identified in tilapia in both our previous (Liang et al., 2022) and present study (Figure 1B), suggesting the potential presence of the LAT signalosome in this early vertebrate species. Furthermore, the tilapia components exhibited conserved functional domain organization analogous to mouse homologs (Figure 1B). Notably, the key components of the tilapia LAT signalosome showed high tertiary structural congruence with their mouse counterparts (Figure 1C). Phylogenetic trees were constructed to further investigate the evolutionary relationships of the LAT signalosome components. Similar to ITK in our previous study (Liang et al., 2022), all LAT signalosome components from tilapia formed distinct clusters with their counterparts from diverse teleost species (Figure 1D, E; Supplementary Figure S1), suggesting close evolutionary distance. Collectively, these observations indicate that Nile tilapias harbor intact and evolutionarily conserved components of the LAT signalosome.

First signal induces activation and formation of LAT signalosome

As the first signal triggered by the TCR/CD3 complex is essential for T-cell activation, we first determined the exact signal during CD3ε mAb-induced T-cell activation in tilapia. Downstream of the TCR, the mRNA levels of early T-cell activation molecules, including LCK and ZAP-70, were significantly increased in the spleen lymphocytes upon CD3ε mAb activation (Figure 2A). Furthermore, phosphorylation of these molecules was concordantly enhanced, as observed in our previous study (Li et al., 2021). First signal-induced T-cell activation lead to an increase in the transcriptional levels of LAT (Figure 2B), as well as ITK, GRB2, SLP-76, GADS, VAV1, and PLC-γ1 (Figure 2C), suggesting activation of the LAT signalosome components downstream of TCR signaling. In mammalian systems, engagement of the TCR typically results in the interaction of LAT with other signaling components to initiate a downstream cascade. Thus, we next investigated the constitutive associations of the LAT signalosome in tilapia. As revealed by the Co-IP assay, tilapia LAT directly interacted with ITK (Figure 2D), GRB2 (Figure 2E), and VAV1 (Figure 2F), but showed no direct association with SLP-76, GADS, or PLC-γ1 (Figure 2G–I). These findings suggest that while the roles of SLP-76, GADS, and PLC-γ1 in the LAT signalosome remain uncertain, LAT, ITK, GRB2, and VAV1 are integral components for intracellular signaling transduction in tilapia T cells. Overall, these results underscore the role of the first signal in modulating LAT signalosome activation and formation in tilapia T cells.

First signal activates T cell canonical pathways in tilapia

Next, we investigated whether the first signal regulates T-cell activation in tilapia. Downstream of TCR signaling, several canonical pathways, such as NF-κB and MAPK/ERK, are essential prerequisites for orchestrating T-cell-mediated adaptive immunity (Gorentla & Zhong, 2012). Here, upon CD3ε mAb-induced T-cell activation, there was a significant increase in the transcription levels of IKKα/β, IκBα, and NF-κB p65 in the spleen lymphocytes (Figure 3A). Furthermore, concurrent enhancements were observed in the phosphorylation of IKKα/β, IκBα, and NF-κB p65, and degradation of IκBα (Figure 3B), suggesting robust activation of the NF-κB pathway. In line with these findings, the MAPK/ERK pathway was also activated downstream of the first signal, as evidenced by the elevated transcription and phosphorylation of MAPK/ERK components (i.e., c-Raf, MEK1/2 and ERK1/2) and transcription factor c-Fos upon CD3ε mAb stimulation (Figure 3C, D). Previous studies have indicated that the first signal activates the AKT-mTOR pathway through the NF-κB and MAPK/ERK axis in mammals (Gorentla et al., 2011). Our results revealed that this regulation is not exclusive to mammals. Notably, we observed a marked increase in the mRNA and phosphorylation levels of mTOR components, including AKT, S6, and 4E-BP1, in tilapia during CD3ε mAb-induced T-cell activation (Figure 3E, F). Thus, these results suggest that the first signal activates multiple canonical pathways crucial for the activation of T cells in tilapia.

CD28-mediated co-stimulatory signal initiates AKT-mTORC1 pathway in tilapia

In addition to the first signal, CD28-mediated co-stimulatory signaling is also essential for T-cell activation, proliferation,

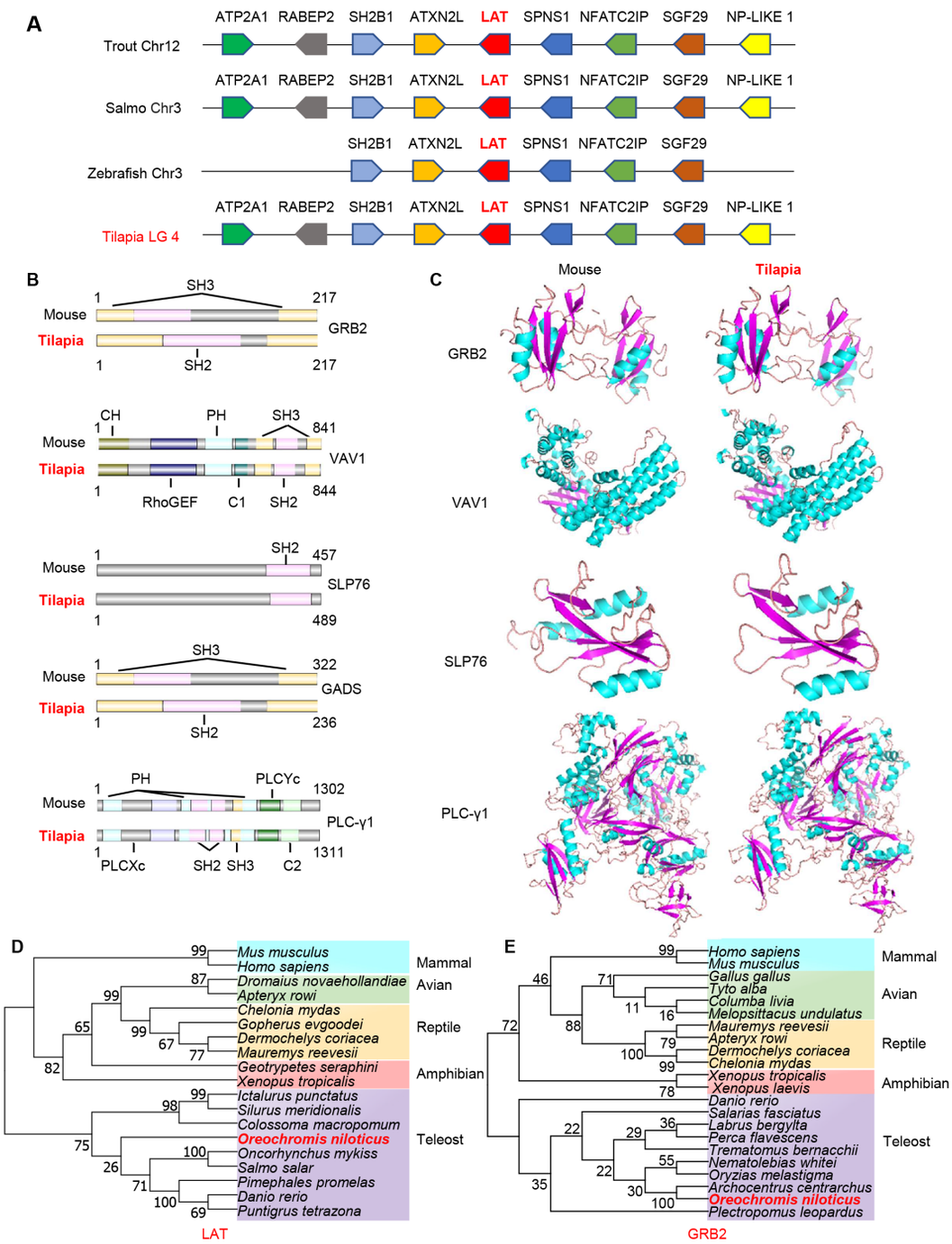

Figure 1 Evolutionary conservation of LAT signalosome molecules in tilapia

A: Synteny and chromosomal location of LAT gene in teleosts. B: Comparative analysis of domain organization of LAT signalosome components in Nile tilapia and mouse. C: Tertiary structure of LAT signalosome molecules in Nile tilapia and mouse were predicted by SWISS-MODEL. D, E: Phylogenetic trees of LAT and GRB2 were constructed using MEGA v7.0 with the neighbor-joining method. Bootstrap values for 1 000 replicates (%) are indicated on each branch.

differentiation, and function (Marinari et al., 2004). Through immunofluorescence and flow cytometry assays, we detected CD28 on the surface of the tilapia lymphocytes (Figure 4A), with tilapia CD28$^+$ cells accounting for approximately 30% and 20% of the lymphocytes in the spleen and head kidney, respectively (Figure 4B). To confirm the expression of CD28 in tilapia T cells, the spleen and head kidney lymphocytes were stained with CD28 and CD3ε mAbs for flow cytometry. Results showed that CD28 was predominantly expressed in the CD3$^+$ T-cell population in both the spleen and head kidney (Figure 4C), as supported by the co-localization of CD28 and CD3 based on immunofluorescence assay (Figure 4D). To further investigate whether the CD28-mediated co-stimulatory signal regulates T-cell activation, the spleen lymphocytes were stimulated with CD28 mAbs. Compared to the unstimulated control, CD28 and CD28 mAb ligation enhanced both the transcription and phosphorylation of AKT (Figure 4E, F), subsequently activating downstream mTORC1 signaling, as evidenced by the increased transcription and phosphorylation of S6 and 4E-BP1 (Figure 4E, F). These results suggest that the CD28-mediated co-stimulatory signal may regulate T-cell responses in tilapia via AKT-mTORC1 pathway initiation.

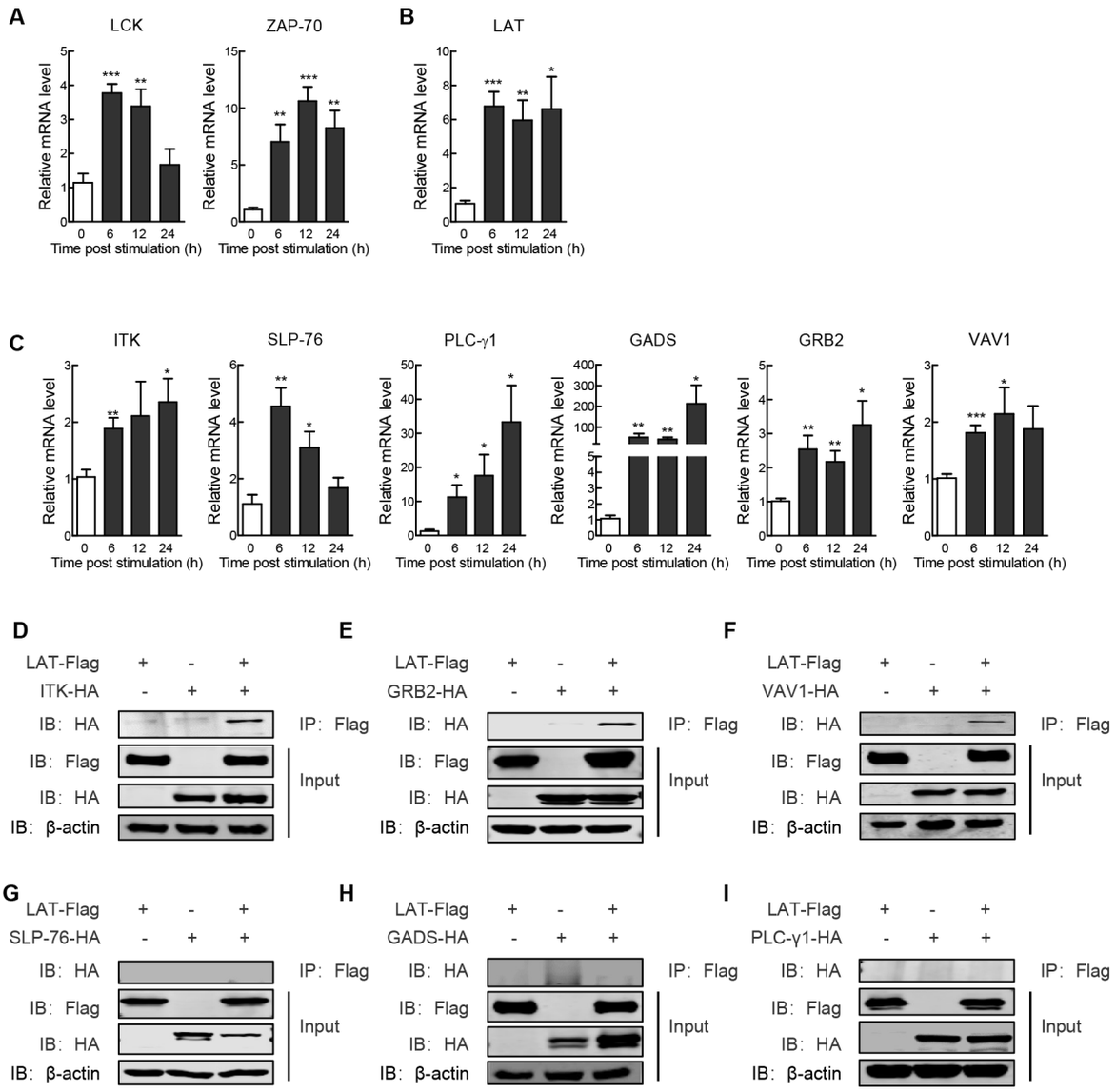

Figure 2 Expression patterns and interactions of tilapia LAT signalosome molecules

A–C: Relative mRNA levels of indicated molecules in spleen lymphocytes upon CD3ε mAb stimulation, as determined by qRT-PCR, $n \geq 4$. D–I: Interactions of Nile tilapia LAT with ITK (D), GRB2 (E), VAV1 (F), SLP-76 (G), GADS (H), and PLC-γ (I), as determined by Co-IP and western blot assay. Experiments were conducted at least twice independently. *: $P<0.05$; **: $P<0.01$; ***: $P<0.001$.

Dual signals are critical for full activation of T cells in tilapia

We next examined the essential role of dual signals in T-cell activation of tilapia. Stimulation of spleen lymphocytes with CD3ε and CD28 mAbs resulted in a pronounced increase in the phosphorylation of ERK1/2 and its nuclear translocation (Figure 5A). A similar trend was observed for the phosphorylation and nuclear translocation of S6 (Figure 5B). These events, related to MAPK/ERK and mTORC1 activation, suggest the potential involvement of dual signals in promoting T-cell activation of tilapia. Although stimulation with either CD3ε or CD28 mAbs alone can induce mRNA expression of T-cell activation transcription factors NFAT1 and c-Fos, combined stimulation with CD3ε and CD28 mAbs led to much higher expression (Figure 5C). Stimulation with the first signal, but not the second signal, also increased the transcription of T-cell activation markers CD122 and CD44 in the spleen lymphocytes (Figure 5D), whereas activation of both signals resulted in more pronounced up-regulation (Figure 5D). Thus, these observations suggest that full T-cell activation requires the synergistic cooperation of dual signaling. To assess the effects of the first signal, co-stimulatory signal, and dual signals on tilapia T cell proliferation, CFSE-labeled spleen lymphocytes were stimulated with CD3ε, CD28, or CD3ε plus CD28 mAbs *in vitro*. At 48 h after stimulation, synergistic CD3ε and CD28 mAbs treatment induced significant $CD3^+$ T cell proliferation, while neither the CD3ε nor αCD28 mAbs alone triggered such proliferation (Figure 5E), highlighting the indispensable role of both the first and co-stimulatory signals in T-cell proliferation in tilapia. Taken together, these findings indicate that the dual signals are essential for complete activation and proliferation of tilapia T cells.

Dual signals promote T-cell function and triggers T-cell apoptosis

As the dual signals triggered T-cell activation and proliferation, we further explored their potential roles in regulating tilapia T-cell function. Dual signal stimulation induced high mRNA expression of IL-2 and tumor necrosis factor alpha (TNF-α) (Figure 6A), critical factors in T-cell proliferation, survival, and

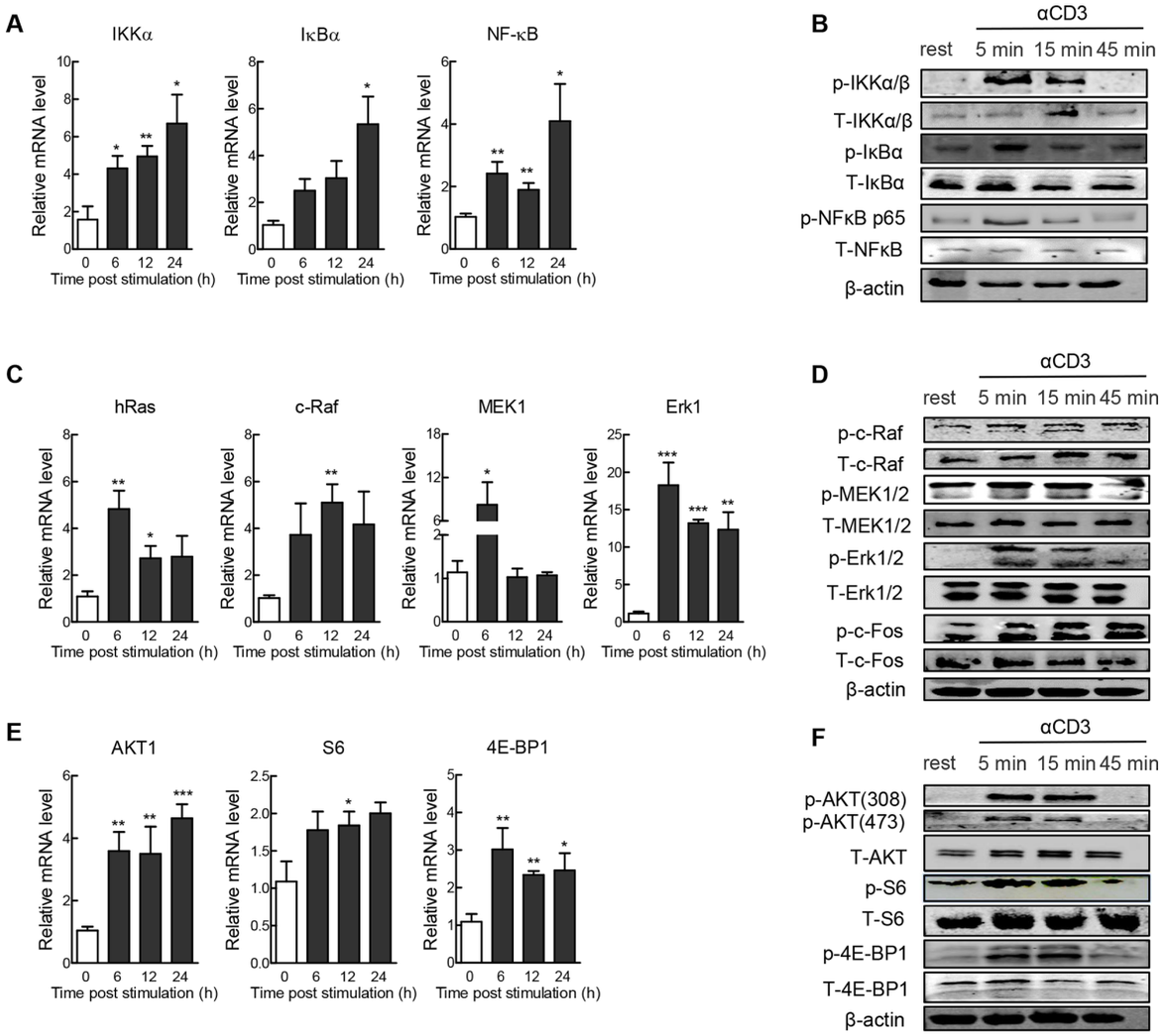

Figure 3 Activation of canonical T-cell pathways by first signal in tilapia

A, B: mRNA and phosphorylation levels of NF-κB signaling components in spleen (A) or head-kidney (B) leukocytes following CD3ε mAb stimulation at indicated times. C, D: mRNA and phosphorylation levels of MAPK/ERK signaling components in spleen (C) or head-kidney (D) leukocytes following CD3ε mAb stimulation at indicated times. E, F: mRNA and phosphorylation levels of PI3K-AKT signaling components in spleen (E) or head-kidney (F) leukocytes following CD3ε mAb stimulation at indicated times. Experiments were conducted at least twice independently. *: P<0.05; **: P<0.01; ***: P<0.001.

function (Smith-Garvin et al., 2009). Given the crucial roles of both helper and cytotoxic T cells in T-cell function and infection elimination, we evaluated the immune responses related to helper and cytotoxic T cells in lymphocytes upon CD3ε plus CD28 mAb stimulation. Compared to the unstimulated control, dual signal-induced T-cell activation resulted in a marked up-regulation in the mRNA levels of T-bet and GATA3, key factors driving the differentiation of $CD4^+$ T cells towards Th1 and Th2 phenotypes (Figure 6B). The transcript levels of cytotoxic genes perforin A and granzyme B, were also significantly induced (Figure 6C). Additionally, synergistic CD3ε and CD28 mAb stimulation increased the expression of apoptosis-related caspase genes, including caspase-3, caspase-6, caspase-8, and caspase-9 (Figure 6D). This resulted in an increase in $CD3^+$ T cell apoptosis (Figure 6E), suggesting that the dual signals triggered activation-induced apoptosis in tilapia T cells. Overall, our findings support the notion that dual signal cooperation is essential for T-cell function and plays a potential role in T-cell apoptosis to maintain immune homeostasis in tilapia.

DISCUSSION

In response to the variable and complex microbial environment, teleosts have evolved a complete T-cell-mediated adaptive immune system based on their innate immune system (Dickerson & Findly, 2017; Lu & Chen, 2019; Ren et al., 2019; Wilson, 2017). Recent studies on the identification of multiple T-cell subpopulations and response patterns after infection strongly underpin the indispensable role of T-cell immunity in fish (Ai et al., 2022; Tafalla et al., 2016; Yamaguchi et al., 2019). To date, however, it remains unclear whether the “two-signal hypothesis” described in mammals also governs the T-cell response in fish. Using the tilapia CD3ε and CD28 mAbs previously developed in our laboratory, we examined the impact of the first and second signals on T-cell activation in teleosts.

T-cell activation is initiated by complex intercellular interactions. Assembly of the CD3-TCR complex is essential for recognition of the MHC-antigenic peptide complex and transmission of TCR signals (Gorentla & Zhong, 2012; Hara et al., 1985; Smith-Garvin et al., 2009). CD3 aids in stabilizing

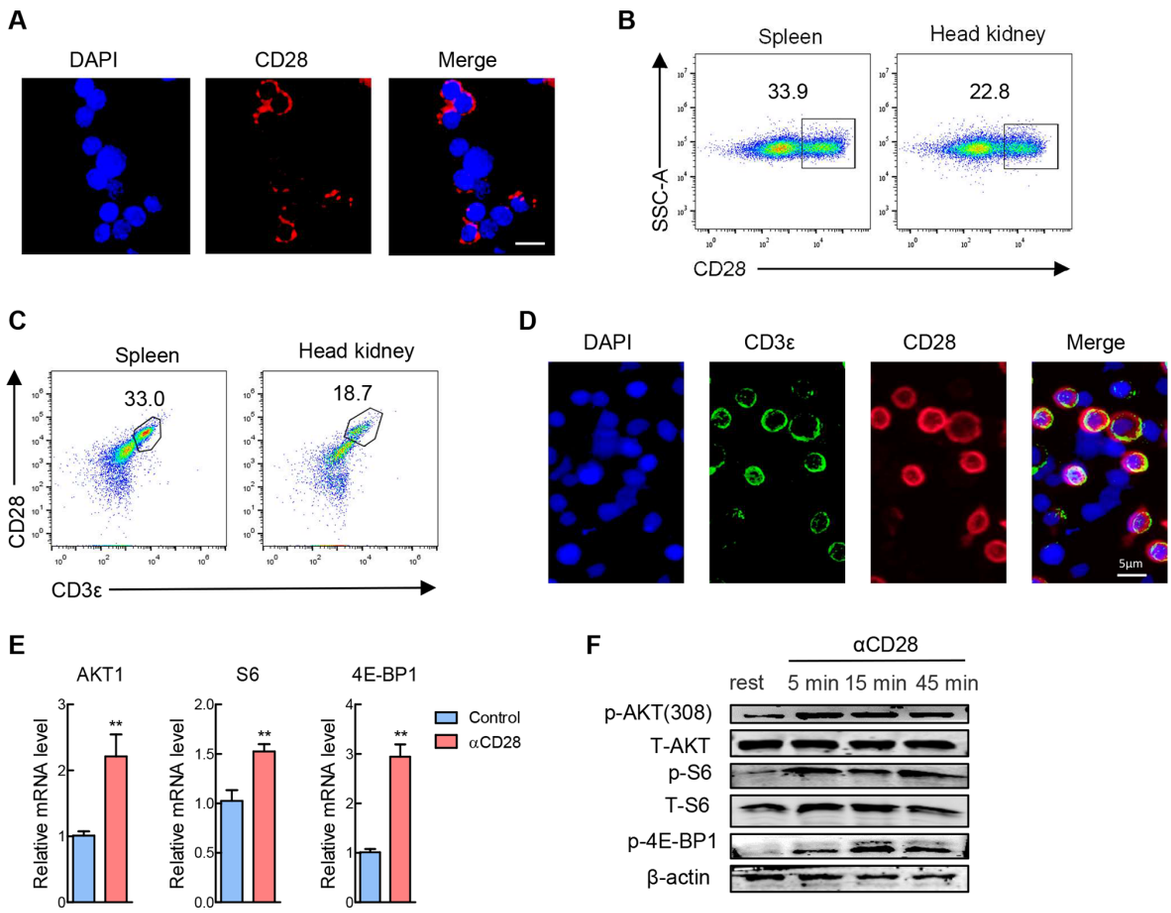

Figure 4 CD28 mAb stimulation induces AKT-mTORC1 signaling in tilapia

A: Localization of CD28 in spleen lymphocytes detected by confocal microscopy. B: $CD28^+$ lymphocytes in spleen and head kidney detected by flow cytometry. C: $CD3\varepsilon^+CD28^+$ lymphocytes in spleen and head kidney detected by flow cytometry. D: Localization of CD3εCD28 in spleen lymphocytes detected by confocal microscopy. E: mRNA levels of mTORC1 signaling components determined 12 h after spleen lymphocytes were stimulated with CD28 mAb, $n \geq 4$. F: Phosphorylation levels of mTORC1 signaling components determined after head-kidney leukocytes were stimulated with CD28 mAbs. Experiments were conducted at least twice independently. **: $P<0.01$.

the immunoreceptor tyrosine activation motif in its cytoplasmic region and transmitting TCR activation signals, triggering a kinase activation cascade to promote T-cell activation (Alcover & Alarcón, 2000; Kuhns et al., 2006). In mammals, functional mAbs against CD3ε chains have been developed and used to induce T-cell activation *in vitro*, providing a valuable tool for investigating T-cell-mediated immune mechanisms (Woodle et al., 1991). While CD3 antibodies have been established in various teleosts, no antibodies have been generated for stimulating T-cell activation, with previous teleost studies relying on PHA or PMA plus ionomycin stimulation to induce T-cell activation *in vitro* (Miyazawa et al., 2018; Mu et al., 2022; Tang et al., 2017). In the present study, Nile tilapia T-cell activation was successfully induced *in vitro* using CD3ε mAbs. The first signal mimicked by CD3ε mAbs induced LCK and ZAP-70 activation, implying the formation of TCR signaling equivalent to that observed in mammals (Gorentla & Zhong, 2012). Downstream of ZAP-70, LAT signalosome formation through the cooperative interactions of LAT with other molecules is crucial for T-cell responses (Liu et al., 2020a). Upon TCR engagement, the interactions of LAT with GADS, GRB2, and PLC-γ1 are indispensable for T-cell development (Zhu et al., 2003). Furthermore, the multivalent adaptor protein SLP-76 interacts with LAT and GADS to stabilize their persistent signaling clusters for T-cell activation (Bunnell et al., 2006). To date, however, no previous studies have identified the LAT signalosome in early vertebrates, particularly teleosts. In our research, we not only confirmed the presence of potential LAT signalosome components in tilapia, including LAT, ITK, GRB2, VAV1, SLP-76, GADS, and PLC-γ1, but also demonstrated that their expression was induced by activation of the first signal. Of these, LAT cooperatively interacted with ITK, GRB2, and VAV1 to transmit TCR signals. Intriguingly, the binding of tilapia LAT to SLP-76, GADS, or PLC-γ1 was not detected by Co-IP assay, implying the possibility of functional differences between teleosts and mammals. However, considering that Co-IP assays based on overexpression systems cannot exclude transient or indirect binding, the detailed contribution of these molecules in forming LAT signalosomes remains unclear. Thus, further study is required to elucidate whether SLP-76, GADS, and PLCγ1 are involved in the formation of LAT signalosomes to regulate T-cell activation in tilapia.

Activation of PLC-γ1 may facilitate the response of components within the NF-κB and MAPK/ERK pathways (Nishibe et al., 1990). In mammals, accumulating evidence suggests that TCR engagement may drive NF-κB signaling, which not only governs T-cell activation, proliferation, and cytokine release, but also T-cell development, survival, and function (Hayden & Ghosh, 2011; Li & Verma, 2002). Our previous studies on tilapia revealed that PHA or combined PMA plus ionomycin stimulation activated the NF-κB and

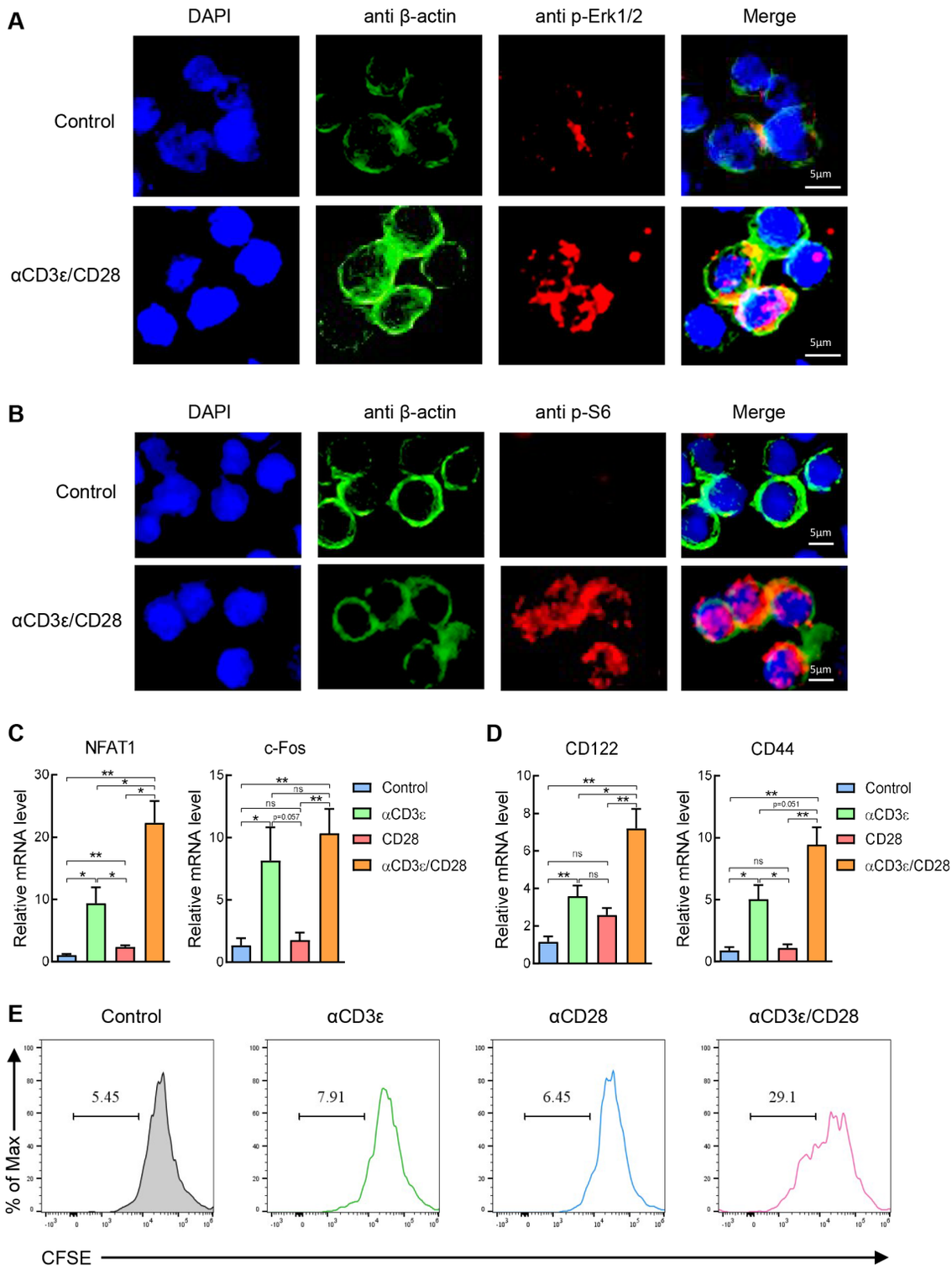

Figure 5 Dual signals trigger full activation of T cells in tilapia

A, B: Phosphorylation of ERK1/2 or S6 examined by confocal microscopy after spleen lymphocytes were stimulated with CD3ε plus CD28 mAbs for 12 h. C, D: mRNA levels of indicated molecules determined 12 h after spleen lymphocytes were stimulated with CD3ε mAbs, CD28 mAbs, or CD3ε plus CD28 mAbs, n≥4. E: Proliferation of spleen lymphocytes examined by flow cytometry 48 h after cells were stimulated with CD3ε mAbs, CD28 mAbs, or CD3ε plus CD28 mAbs, with unstimulated lymphocytes used as a control. Experiments were conducted at least twice independently. ns: No significance; *: P<0.05; **: P<0.01; ***: P<0.001.

MAPK/ERK pathways, thereby orchestrating the activation, proliferation, differentiation, and function of T cells (Wei et al., 2020, 2021). The present study supports our previous findings, asserting the involvement of the NF-κB and MAPK/ERK signaling pathways in T-cell activation after CD3ε mAb stimulation. Downstream of MAPK/ERK signaling, the transcription factor c-Fos, an AP-1 component member, plays an important role in the regulation of numerous processes, including cell migration, proliferation, differentiation, apoptosis, and angiogenesis (You et al., 2016). In fish species such as

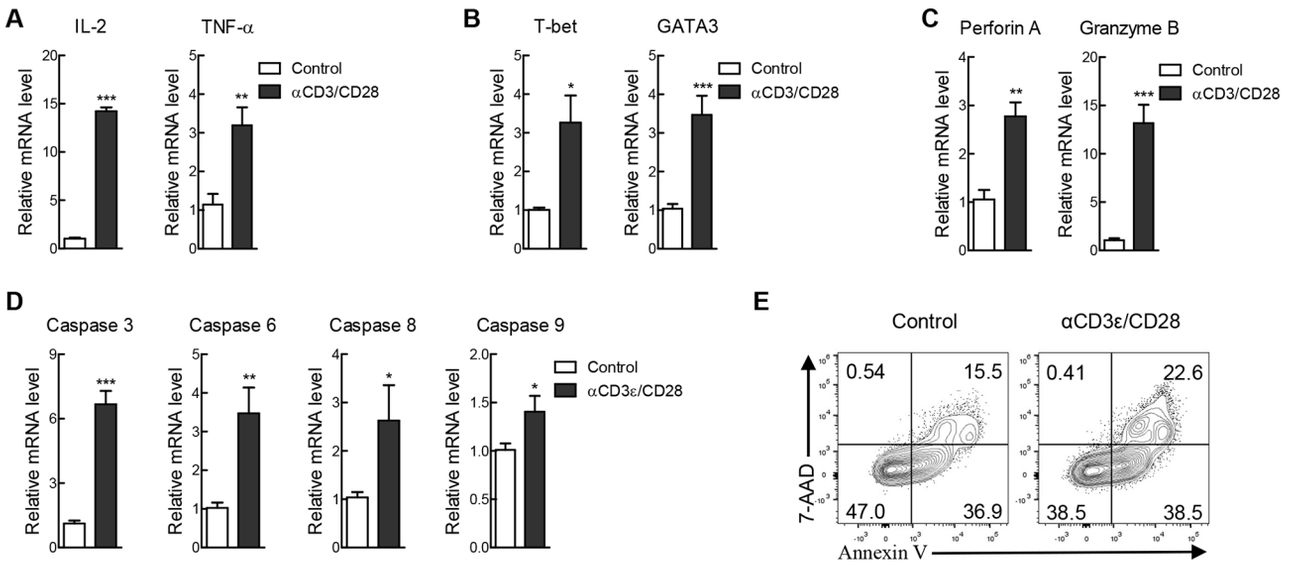

Figure 6 Dual signal-induced T-cell activation is accompanied by cytotoxic responses and apoptosis

A–D: mRNA levels of indicated molecules in spleen lymphocytes examined 12 h after cell stimulation with CD3ε plus CD28 mAbs, $n \geq 4$. E: Representative contour plot of Annexin V and 7AAD staining of CD3ε$^+$ T cells 12 h after spleen lymphocytes were stimulated with CD3ε plus CD28 mAbs. Experiments were conducted at least twice independently. *: $P<0.05$; **: $P<0.01$; ***: $P<0.001$.

Liza haematocheila, c-Fos expression can be strongly induced by poly I:C or *Lactococcus garvieae* stimulation, suggesting the possible involvement of c-Fos in the fish immune response (Janson et al., 2019). Consistent with this finding, we demonstrated the response of c-Fos to TCR signaling in tilapia T cells, highlighting its potential role in regulating T-cell biological processes. Notably, the PI3K/AKT signaling pathway was similarly induced upon CD3ε mAb stimulation. Drawing parallels from mammalian systems, we theorize that heightened Ras activity may modulate PI3K/AKT signaling (Chappell et al., 2011), although this hypothesis requires further investigation in future studies. Nevertheless, our results support the notion that the first signal plays a vital role in initiating T-cell activation in teleosts.

Over the last fifty years, extensive research has explored many co-stimulatory receptors (CD28–CD80/CD86) on the surface of T cells, which spatiotemporally regulate the biology of these cells (Esensten et al., 2016; June et al., 1990). The binding of the key co-stimulatory receptor CD28 to the B7 family molecules CD80/CD86 generates co-stimulatory signals that promote T-cell activation, proliferation, survival, and cytokine secretion (Esensten et al., 2016; June et al., 1990; Tavano et al., 2006). Positive stimulatory effects of CD28 molecules have been reported in several teleost species, including rainbow trout, grass carp, and half-smooth tongue soles (Hu et al., 2012; Lu et al., 2022; Zhang et al., 2009). For instance, the cytoplasmic tail of trout CD28 mediates ERK phosphorylation, and the chimeric receptor formed by the fusion of the extracellular structure of human CD28 with the cytoplasmic tail of rainbow trout CD28 promotes TCR-induced IL-2 production in human T-cell lines, indicating that trout CD28 is indeed a positive co-stimulatory factor (Bernard et al., 2006). In the present study, we found that tilapia CD28 was highly expressed on the surface of T cells isolated from the spleen and head kidney. Furthermore, the mTORC1 pathway was strongly activated by ligation of CD28 mAbs, suggesting that tilapia CD28 is involved in T-cell activation as a positive co-stimulator.

Recent mammalian studies have shown that full T-cell activation, proliferation, survival, and cytokine release require synergistic stimulation by the first signal and CD28-mediated co-stimulatory signals (Esensten et al., 2016; June et al., 1990). Our results also showed that dual signaling by CD3ε plus CD28 mAb stimulation strongly induced mTORC1 and ERK pathway activation in T cells, demonstrating the importance of dual signaling for T-cell activation. In the absence of CD28-mediated co-stimulatory signals, T cells fail to achieve full activation, instead entering an anergic state categorized by an inability to proliferate and become hypo-responsive (Esensten et al., 2016; Gimmi et al., 1993). Similarly, our results showed that synergistic stimulation by CD3ε plus CD28 mAbs induced tilapia T-cell proliferation, while stimulation by CD3ε or CD28 mAbs alone did not induce the same proliferation, implying that tilapia T-cell proliferation requires synergistic stimulation by dual signaling. This finding may be related to the expression of IL-2, a key T cell growth factor, as the absence of CD28 signaling in mammals impairs the expression of IL-2 and further affects the expression of various effector molecules (Coppola et al., 2020). Similarly, our study showed that tilapia T cells synergistically stimulated by dual signaling robustly induced IL-2 expression, further promoting T-cell differentiation. Notably, previous research has shown that combined treatment with CD28 antibodies and PHA can stimulate the proliferation of flounder peripheral blood leukocytes and increase the transcription of cytokines IL-2, TNF-α, IFN-γ, and IL-6 (Xing et al., 2021). Remarkably, in half-smooth tongue soles, the binding of CD28 antibodies alone significantly induces kidney lymphocyte proliferation, suggesting considerable differences among teleost CD28 molecules (Hu et al., 2012). In addition, our results emphasized that dual signaling synergism induced T-cell apoptosis in tilapia, a phenomenon likely attributed to activation-induced apoptosis as opposed to apoptosis resulting from CD28 deficiency (Green et al., 2003).

In summary, our study demonstrated that T-cell-mediated adaptive immune responses in tilapia are regulated by synergy between the CD3/TCR-mediated first signal and CD28-mediated co-stimulatory signal. Notably, dual signaling

is required for complete T-cell activation, subsequently inducing T cells to enter a proliferative and responsive state. Moreover, co-stimulation by dual signaling plays a critical role in activation-induced T-cell apoptosis as a mechanism for immune homeostasis maintenance. Thus, our results suggest that dual signaling is essential for T-cell functions in response to variable microbial environments. To the best of our knowledge, this study is the first to describe dual signaling-mediated T-cell responses in teleosts. Overall, these findings provide a robust basis for identifying T-cell immune mechanisms in teleosts and offer a novel perspective for understanding the evolution of the adaptive immune system.

SUPPLEMENTARY DATA

Supplementary data to this article can be found online.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

W.L. designed and performed the experiments, analyzed the data, and drafted the manuscript. Kang L., H.G., Kunming L., J.Z., Q.Z., and X.J. performed the experiments. J.Y. and X.W. acquired funding, conceived the project, designed the experiments, analyzed the data, and drafted the manuscript. All authors read and approved the final version of the manuscript.

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