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Evolutionarily conserved IL-27β enhances Th1 cells potential by triggering the JAK1/STAT1/T-bet axis in Nile tilapia

Ming Geng [a], Kang Li [a,*], Kete Ai [a], Wei Liang [a], Jialong Yang [a,b], Xiumei Wei [a,*]

[a] State Key Laboratory of Estuarine and Coastal Research, School of Life Sciences, East China Normal University, Shanghai 200241, China
[b] Laboratory for Marine Biology and Biotechnology, Qingdao National Laboratory for Marine Science and Technology, Qingdao 266237, China

A R T I C L E I N F O

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A B S T R A C T

As a pleiotropic cytokine in the interleukin (IL)-12 family, IL-27β plays a significant role in regulating immune cell responses, eliminating invading pathogens, and maintaining immune homeostasis. Although non-mammalian IL-27β homologs have been identified, the mechanism of whether and how it is involved in adaptive immunity in early vertebrates remains unclear. In this study, we identified an evolutionarily conserved IL-27β (defined as OnIL-27β) from Nile tilapia (*Oreochromis niloticus*), and explored its conserved status through gene collinearity, gene structure, functional domain, tertiary structure, multiple sequence alignment, and phylogeny analysis. IL-27β was widely expressed in the immune-related tissues/organ of tilapia. The expression of OnIL-27β in spleen lymphocytes increased significantly at the adaptive immune phase after *Edwardsiella piscicida* infection. OnIL-27β can bind to precursor cells, T cells, and other lymphocytes to varying degrees. Additionally, IL-27β may be involved in lymphocyte-mediated immune responses through activation of Erk and JNK pathways. More importantly, we found that IL-27β enhanced the mRNA expression of the Th1 cell-associated cytokine IFN-γ and the transcription factor T-bet. This potential enhancement of the Th1 response may be attributed to the activation of the JAK1/STAT1/T-bet axis by IL-27β, as it induced increased transcript levels of JAK1, STAT1 but not TYK2 and STAT4. This study provides a new perspective for understanding the origin, evolution and function of the adaptive immune system in teleost.

1. Introduction

Interleukin (IL)-27 is a heterodimer cell factor composed of Epstein-Barr virus (EBV) induced gene 3 (Ebi3, also known as IL-27β) and IL-27p28, belonging to the IL-6/IL-12 family of cytokines [1,2]. The receptor consists of gp130 and IL-27 receptor alpha chains (IL-27Rα, also named TCCR or WSX1) [3]. IL-27 acts on a variety of cell types including $CD4^+$ T cells, $CD8^+$ T cells, B cells, dendritic cells, macrophages, non-hematopoietic cells and natural killer cells (NK cells) [3]. In particular, T cells have been intensively studied, revealing that different T cell subsets have unique responses to IL-27 [4]. Remarkably, binding between IL-27 and IL-27R is critical for the early initiation of Th1 responses, while IL-27 enhances Th1 differentiation and is associated with $CD8^+$ T cell activation and enhanced humoral responses in specific environments [4,5]. However, IL-27 also has powerful inhibitory properties, and mice lacking IL-27-mediated signaling have excessive inflammatory responses in cases of infection or autoimmunity [6]. However, the immunosuppressive effect of IL-27 is achieved by blocking the development of Th17 cells, promoting Treg cells differentiation and inducing IL-10 production [7,8].

As a pleiotropic cytokine, IL-27 provides distinctive support for tissue repair and inflammatory regulation by promoting or inhibiting T cell response, and is related to autoimmunity and infectious diseases [4]. IL-27 is usually up-regulated during inflammation and tissue damage and plays a role by promoting Th1 differentiation, and is involved in the inhibition and treatment of enteritis, pneumonia, chronic immune thrombocytopenia, and aplastic anemia [9–12]. T cells produce IL-27 in synergy with IFN-γ; for example, compared to healthy controls, psoriasis patients had higher serum levels of IL-27, which correlated with disease severity and serum IFN-γ levels [13]. IL-27 is positively correlated with IFN-γ in synovial fluid of patients with rheumatoid arthritis, inducing Th1 differentiation and inhibiting Th17 cell development or migration [14]. Despite the role of IL-27 in promoting Th1 differentiation, WSX-1-deficient mice infected with various pathogens have shown that IL-27 inhibits IL-2 production by $CD4^+$ T cells [15]. Therefore, IL-27 signaling prevents T-cell overactivity and limits inflammatory

* Corresponding authors.
E-mail addresses: kanglbio@163.com (K. Li), xmwei@bio.ecnu.edu.cn (X. Wei).

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responses to maintain immune homeostasis [16]. Paradoxically, in the context of cancer therapy, IL-27 may be used as a Treg inhibitor, inhibiting IL-2-induced Treg expansion in tumors and thus promoting anti-tumor immune responses, suggesting that the pro-inflammatory or anti-inflammatory features of IL-27 is determined by the particular immune microenvironment [4,17].

IL-27p28 binds to IL-27β to form a heterodimer of IL-27, which induces a signaling cascade by linking to IL-27Rα and gp130 composed of IL-27R and is involved in the Janus activated kinase/signal transducer and activator of transcription (JAK/STAT) pathway and mitogen-activated protein kinase (MAPK) signaling [18,19]. Notably, IL-27 binding to its receptor led to phosphorylation of JAK1/2 or tyrosine kinase 2 (TYK2), which in turn induced activation of STAT1, key transcription factors regulating transcription of T-bet and IFN-γ, implicating a potent association between IL-27 signaling and Th1 cells [3,20]. And the accumulated evidence now also demonstrates a critical role of IL-27 signaling in the early regulation of Th1 initiation, inducing proliferation of infantile $CD4^+$ T cells and acting synergistically with IL-12 in IFN-γ production [21]. Naïve Th cells were induced with the expression of T-bet and slowly differentiated into Th1 cells in response to IFN-γ and IL-12 stimulation [22]. In addition, IL-27 increased the surface expression of IL-12Rβ2 and the production of IL-12-dependent IFN-γ [21,23]. IL-27 can also antagonize the blocking effect of IL-4 on the differentiation of Thl cells and promote the differentiation of Thl cells by inhibiting the expression of GATA-3 [21]. Simultaneously, IL-27 promotes Th1 cell infiltration in preterm labor by inducing the secretion of CXCL9, CXCL10, and CXCL11 and the activation of JAK2/STAT1/STAT3 [24].

IL-27β was first identified from B cell line transformed by EBV virus in 1996 [2], and its cell origin, function, signal transduction, and mechanism have been reported successively in human and mouse. Although the cDNA sequences encoding IL-27β homologues have been developed in grass carp, tongue sole, tilapia, fugu, rainbow trout, and zebrafish, the biological function of IL-27β in teleost remains largely unknown [25]. Recent studies on teleost illustrated the expression pattern of IL-27β, it is highly expressed in peripheral immune tissues such as head kidney, spleen, and blood [25,26]. And its expression can be induced by bacteria, viruses, and their analogs. In kidney and spleen tissues of tongue sole (*Cynoglossus semilaevis*), IL-27β expression was up-regulated after induction by *Edwardsiella tarda* and megalocytivirus [25]. Two copies of IL-27β interacting were found in grass carp, and both of them significantly responded to LPS and Poly I:C stimulation in head kidney lymphocytes [27]. Functionally, *C. semilaevis* IL-27β (CsIL-27β) functions as a secreted protein that is secreted into the peripheral blood in a time-dependent manner in response to pathogen stimulation and significantly increases ROS production [25]. In addition, recombinant CsIL-27β, either alone or as a cytokine complex, activates the innate immune response to PBL, thereby enhancing bacterial clearance [25]. Although this evidence indicates the involvement of IL-27 in the immune defense of teleost, the cellular origin of IL-27β, its potential involvement in adaptive immune responses, and downstream signaling are unknown in teleost. Consequently, in this study, we explored the structural features and distribution pattern of IL-27β in Nile tilapia and demonstrated its potential to be involved in adaptive immune responses. Further studies also revealed the target cells of tilapia IL-27β and its mediated downstream signaling pathways, and elucidated the potential association between IL-27β signaling and Th1 cells. Our study will provide a theoretical basis for the lymphocyte mediated adaptive immunity of teleost and a new perspective on the origin and evolution of the adaptive immune system.

2. Materials and methods

2.1. Experimental animals

Nile tilapia (body length:8-10 cm) were purchased from the aquatic farm in Guangzhou, Guangdong Province, China. All fish were reared in the aerated freshwater circulating system at 28 °C and fed with commercial pellets daily. The fish were kept for at least one week before experiments. All fish care and experimental procedures were performed in accordance with the Guide for the Care and Use from Laboratory Animals of the Ministry of Science and Technology of China and were approved by the East China Normal University Experimental Animal Ethics Committee with an approval number AR2021-247. All efforts were made to minimize the suffering of the animals.

2.2. Sequence, structure, and phylogenic analysis

We obtained the OnIL-27β cDNA and amino acid sequence (XP_003442867.1) by using the protein sequences of human and zebrafish IL-27 for BLAST-search against the NCBI (http://www.ncbi.nlm.nih.gov/). The gene structure and gene collinearity of IL-27β were analysed from NCBI and Ensembl database (http://asia.ensembl.org/index.html). The functional domains were obtained by using SMART databases (http://smart.embl-heidelberg.de/). The tertiary structures were predicted by SWISS-MODEL databases (https://swissmodel.expasy.org/) and PyMOL software. Multiple alignments were generated by the Clustal X program. The neighbor-joining (NJ) tree was constructed using MEGA X with 1000 bootstrap replicates. The information of the sequences used for analysis were listed in Table S1.

2.3. Recombinant expression of OnIL-27β

The cDNA fragment encoding OnIL-27β was amplified using a pair of gene-specific primers (Forward: 5′ CGGAATTCGTGGACCTGCTGAGAGGAAC 3′; Reverse: 5′ CCCTCGAGCAGTCTTTTTCTTGGTATTG 3′). After double digestion by restriction enzyme EcoR I and Xho I, it was further connected to His-tag pET28a (+) vector and transformed by *E. coli* BL21 DE3 competent cells (Transgene). The transformed positive monoclonal colonies were cultured in LB medium at 37 °C to OD600 of 0.4-0.6, and added 1 mmol/L β-D-Thiogalactoside (IPTG) into the bacterial solution for 4 h. After ultrasonic treatment in buffer solution, the recombinant His-tagged OnIL-27β (rOnIL-27β) protein was purified by Ni^{2+} adsorption column and eluted with 400 mmol/L imidazole. The purified rOnIL-27β was dialyzed in urea-Tris buffer solution with decreasing gradient at 4 °C. Finally, the purified OnIL-27β was separated by 12 % SDS-PAGE gel for Coomasil bright blue staining.

2.4. Leukocytes isolation

Spleen and head kidney leukocytes were isolated from healthy tilapia by Percoll density gradient centrifugation as our previous report [28]. Briefly, spleen and head kidney tissues of tilapia were collected and lightly ground in Leibovitz L-15 medium (Gibco) before being filtered with nylon gauze. The Percoll (GE Healthcare) was mixed with 10 × PBS at a ratio of 9:1 and diluted to 34 % and 52 % (v/v), respectively, using L-15 medium. 52 % Percoll, 34 % Percoll, and 4 ml spleen and head kidney cell suspension were added into a 15 ml centrifuge tube successively. After centrifugation at 500 g with the lowest acceleration and deceleration at room temperature for 35 min, leukocytes were located between 34 % and 52 % Percoll. The middle layer was sucked and washed twice with L-15 medium for further experiments. According to our previous studies, the vast majority of isolated spleen leukocytes were lymphocytes [28].

2.5. Lymphocytes stimulation

Isolated tilapia spleen lymphocytes were cultured in DMEM complete medium (10% fetal bovine serum and 1% penicillin-streptomycin). Spleen lymphocytes were incubated in D-PBS at 28 °C for 30 min. Then, 1×10^7 lymphocytes were stimulated with 5 μg/mL rOnIL-27β for 15 min, 30 min, 4 h, 12 h, and 24 h. After the reaction was terminated by precooling D-PBS, stimulated cells and control stimulated cells were

collected by centrifugation for further experiments.

2.6. Quantitative real-time RT-PCR (qPCR)

Total RNA was extracted from spleen, head kidney, trunk kidney, liver, intestine, gill and peripheral blood of Nile Tilapia using TRIzol reagent (Invitrogen). The extracted RNA was synthesized into cDNA using reverse transcription kit (Novoprotein). The synthesized cDNA was diluted at 1:30 as the template and β-actin was used as the internal reference gene. The relative mRNA levels of target genes were detected by 2 × NovoStart SYBR qPCR SuperMix Plus (Novoprotein), and the relative expression levels of target genes were calculated by $2^{-\triangle\triangle CT}$ method. The gene-specific primers used in this study are listed in Table S2.

2.7. Bacterial infection

E. piscicida from East China University of Science and Technology (Shanghai, China) was cultured in Tryptic soy broth medium at 37 °C to the exponential stage. Then, the bacteria were centrifuged at 4000 rpm, 4 °C for 5 min to collect, washed twice with PBS and then resuspended, and the concentration of 1×10^6 CFU/mL was diluted. Each tilapia was injected with 200 μL bacterial suspension and the control group was intraperitoneally injected with 200 μL PBS, intraperitoneally. On the 4th and 7th day after infection, four individuals were randomly selected for spleen lymphocytes classification and real-time RT-PCR (qPCR) detection.

2.8. Binding of IL-27β to leukocytes

In order to study the leukocytes types that could bind to IL-27β, head kidney leukocytes were incubated with 1 μg rOnIL-27β on ice for 30 min. After two times of wash with FACS buffer, mouse anti-His antibody (1:1000, BBI life sciences) and Alexa Fluor 647-conjugated goat anti-mouse IgG H&L (1:2000, Abcam) were sequentially incubated on ice away from light for another 30 min. After incubation, wash with FACS buffer twice. The same method was used for the final staining of the samples with anti-tilapia CD3ε monoclonal antibody. Another control group was not incubated with recombinant protein, but with secondary antibody to eliminate the possibility of non-specific binding. The stained cells were suspended in the flow tube with FACS buffer solution and analyzed by flow cytometry.

2.9. Western blot assay

Western blot assay was used to test the phosphorylation levels of indicator protein upon rOnIL-27β stimulation. After stimulation, the protein sample was prepared with NP40 cracking buffer on ice for 30 min, and centrifuged at 13,000 g, 4 °C for 10 min. The protein supernatant was absorbed and placed in 5 × protein sample buffer (Solarbio), and heated in boiling water for 8 min. Protein samples were separated by 12% SDS-PAGE and transferred to nitro cellulose (NC) membrane by electrophoresis transfer system (100 V, 2 h) on ice water. NC membranes was sealed in PBST of 4% defat powder at room temperature for 1 h. Then, the NC membranes were incubated with 1:1,000 diluted primary antibody, including anti-β-actin (Cell Signaling Technology), anti-p-Erk1/2 Thr-202/Tyr-204 (Cell Signaling Technology), and anti-p-JNK Thr183/Tyr185 (Cell Signaling Technology) at 4°C overnight. Then, the NC membranes were incubated with Fluor 800-conjugated goat anti-rabbit IgG H&L Alexa (1:30,000, Cell Signaling Technology) or Alexa Fluor 680-conjugated goat anti-mouse IgG H&L (1:10,000, Abcam) at room temperature for 1 h. After each incubation, the NC film was washed with PBST for 3 times and finally scanned with Odyssey CLx Image Studio.

2.10. Statistical analysis

The difference between the treatment group and the control group was determined by two-tailed Student's t-test. The P-value represents the significant difference (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). The bar chart shows the mean ± standard deviation (SD) of multiple replicates of independent experiments. All data were statistically analyzed and displayed using GraphPad Prism 8.0 software.

3. Results

3.1. Synteny and structural characterization of the IL-27β gene family

As revealed by the genome-wide identification in the NCBI GenBank database, Nile tilapia possesses an IL-27β gene on chromosome LG19. The neighboring genes of IL-27β among different vertebrates were first analyzed by comparative genomics to build the phylogenetic relationship of this cytokine. Both tilapia and fugu IL-27βs are located in a chromosomal fragment composed of *ODF3L2A-IL-27β-CRLF1A* genes (Fig. 1A). In tilapia, YJU2 is upstream of IL-27β, but recombination occurs to some extent in humans, mice, and turtle, and YJU2 is always closely linked to IL-27β. In addition, ODF3L2A upstream of fish IL-27 was replaced by ANKRD24 when it evolved into amphibians.

In order to investigate the structural changes of IL-27β gene during evolution, we predicted and mapped the IL-27β gene structure (Fig. 1B). It showed that tilapia IL-27β contains five exons and four introns like mammals, reptiles, and amphibians, while teleost zebrafish and cartilaginous fish shark contain six exons and five introns. In general, IL-27β has maintained a multi-exon-intron structure during evolution, and the gene structure of IL-27β in tilapia is relatively conserved.

3.2. Structure alignments of OnIL-27β and other homologs

The CDS region of OnIL-27β was 759 bp, encoding 252 amino acids with a predicted molecular mass of 27.25 kDa and a theoretical pI of 9.37. Among them, Met_1 to Ala_{24} encodes a signal peptide; Pro_{37} to Ser_{124}, and Pro_{139} to Gly_{224} constitute two modified fibronectin type III (FN III) domains (Fig. 2A). The structure of these domains is similar to that of the mouse homologous (Fig. 2A). In order to identify whether the functional junction domain, we first performed multiple sequence alignments based on the IL-27β protein sequences of tilapia and vertebrates (Fig. 2B). The results revealed that the IL-27β structure consists of a tandem pair of modified FN III domains named the cytokine-binding domain (CBD), which was evolutionarily conserved when compared with its homologues from other vertebrates (Fig. 2B). In addition, these vertebrates' IL-27βs possessed multiple conserved β-stands and functional motifs, including the N-linked asparagine and a characteristic WSXWS signature motif. More importantly, OnIL-27β also contains partial residues (Phe_{97}, Asp_{210}, and Glu_{159}) that are key binding sites to the role of mammalian IL-27βs in cytokine subunit interactions (Fig. 2B). According to tertiary structure prediction, IL-27β in both tilapia and mice was composed of multiple similar β-stands (Fig. 2C). In general, the functional domains and sites of action of IL-27β are highly similar from early vertebrates to mammals, suggesting the potential possibility for their functional similarity.

3.3. Phylogenetic analysis of OnIL-27β

To further explore the evolutionary relationship between OnIL-27β and its congeners in other species, an adjacent phylogenetic tree was constructed (Fig. 3). OnIL-27β first clustered into a clade with *S. chuatsi* IL-27β, then formed a sister group with a clade formed by *D. rerio* and *E. Lucius* IL-27βs, and finally with mammal, avian, reptile, amphibia, and cartilaginous. The clustering of OnIL-27β and its homologues in other bony fish suggests a close evolutionary relationship, and conforms to its evolutionary status (Fig. 3). In summary, the phylogenetic status,

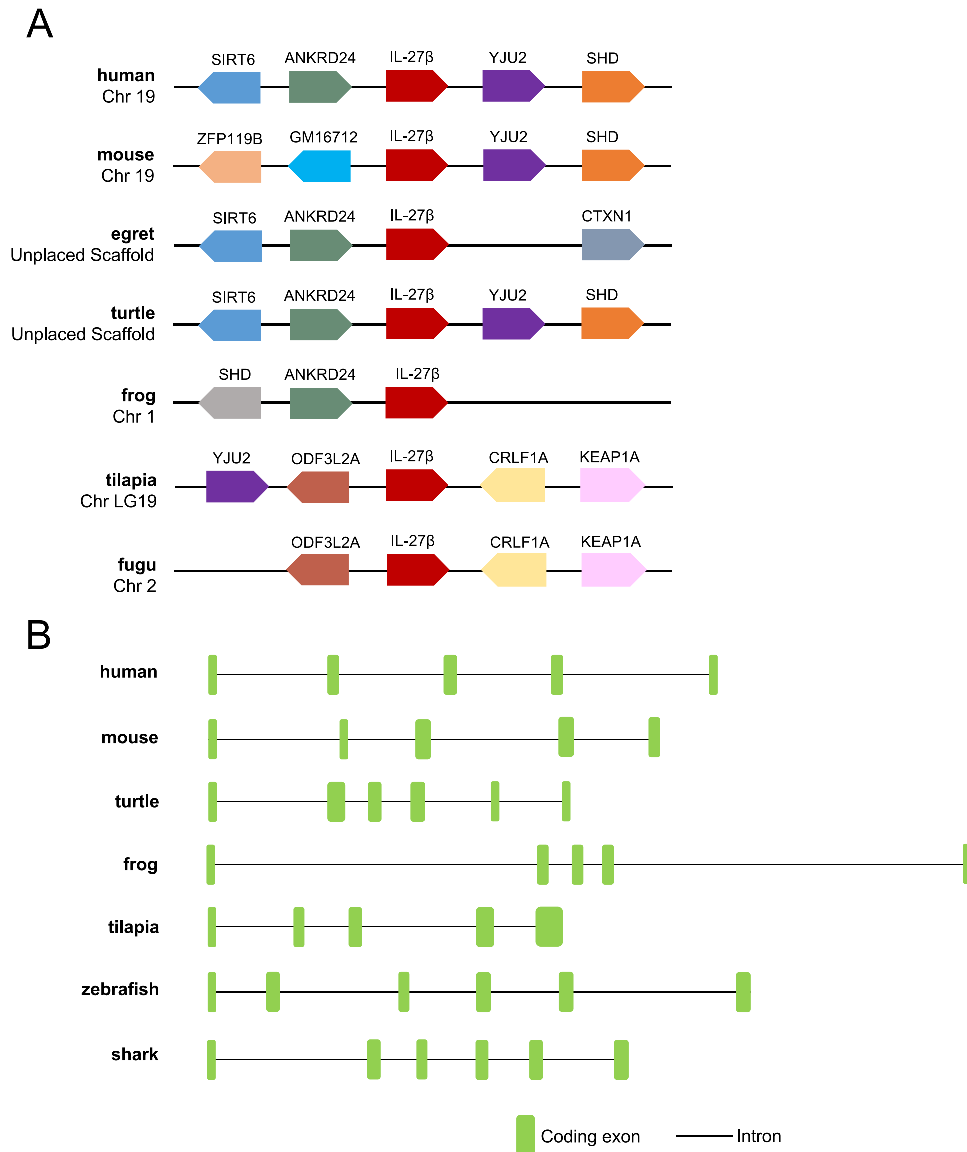

Fig. 1. Gene collinearity and gene structure analysis of IL-27β. (A) Gene collinearity analysis within the IL-27β. The genes are indicated by block arrows, which show their position and orientation in the genome. (B) Comparison of the genomic organizations of IL-27β. The coding-exons and introns are indicated by boxes and lines, respectively.

collinearity, exon-intron composition, functional motif and tertiary structure of OnIL-27β were relatively conserved in the process of evolution. It suggested that OnIL-27β may have similar functions to IL-27βs in higher vertebrates.

3.4. OnIL-27β participates in the lymphocyte-mediated immune response of tilapia

Since the conserved sequence and structure of OnIL-27β suggested a similar immune function to that of mammals, we first examined the distribution pattern of transcript levels of OnIL-27β in immune-related tissues/organ. OnIL-27β was widely distributed in immune-related tissues/organ, including gill, intestine, trunk kidney, head kidney, thymus, liver, peripheral blood and spleen (Fig. 4A). OnIL-27β expression was highest in the spleen, and relatively high abundance was detected in mucosa-associated lymphoid tissues such as the intestine and gill, while relatively low expression was detected in peripheral lymphoid organs such as the head kidney and trunk kidney (Fig. 4A). The extensive distribution of OnIL-27β in immune-related tissues/organ implies its possible involvement in the antimicrobial immune response in tilapia.

To establish the involvement of OnIL-27β in lymphocyte-mediated immune responses, Nile tilapia were infected with *E. piscicida* and the dynamics changes of OnIL-27β transcript levels in spleen lymphocytes were monitored during the primary adaptive immune response phase induced by bacterial infection. Compared with uninfected group, the mRNA level of OnIL-27β was significantly induced on day 4 and 7 post-infection (Fig. 4B). In conclusion, these results indicated that OnIL-27β was involved in the lymphocyte-mediated adaptive immune response in tilapia.

3.5. Development of recombinant OnIL-27β

To investigate the immune function of IL-27β, we prepared the recombinant OnIL-27β protein using a prokaryotic expression system. After IPTG induction, we could clearly find a protein band of about 25 kDa (Fig. 5), and this is consistent with the predicted molecular weight of IL-27β. After purification by Ni^{2+} adsorption column, rOnIL-27β with high purity could be obtained. The protein was placed at 4 °C for gradient dialysis and finally was thoroughly dialysis in PBS (Fig. 5). Subsequently, we used the high-purity and renaturated rOnIL-27 to

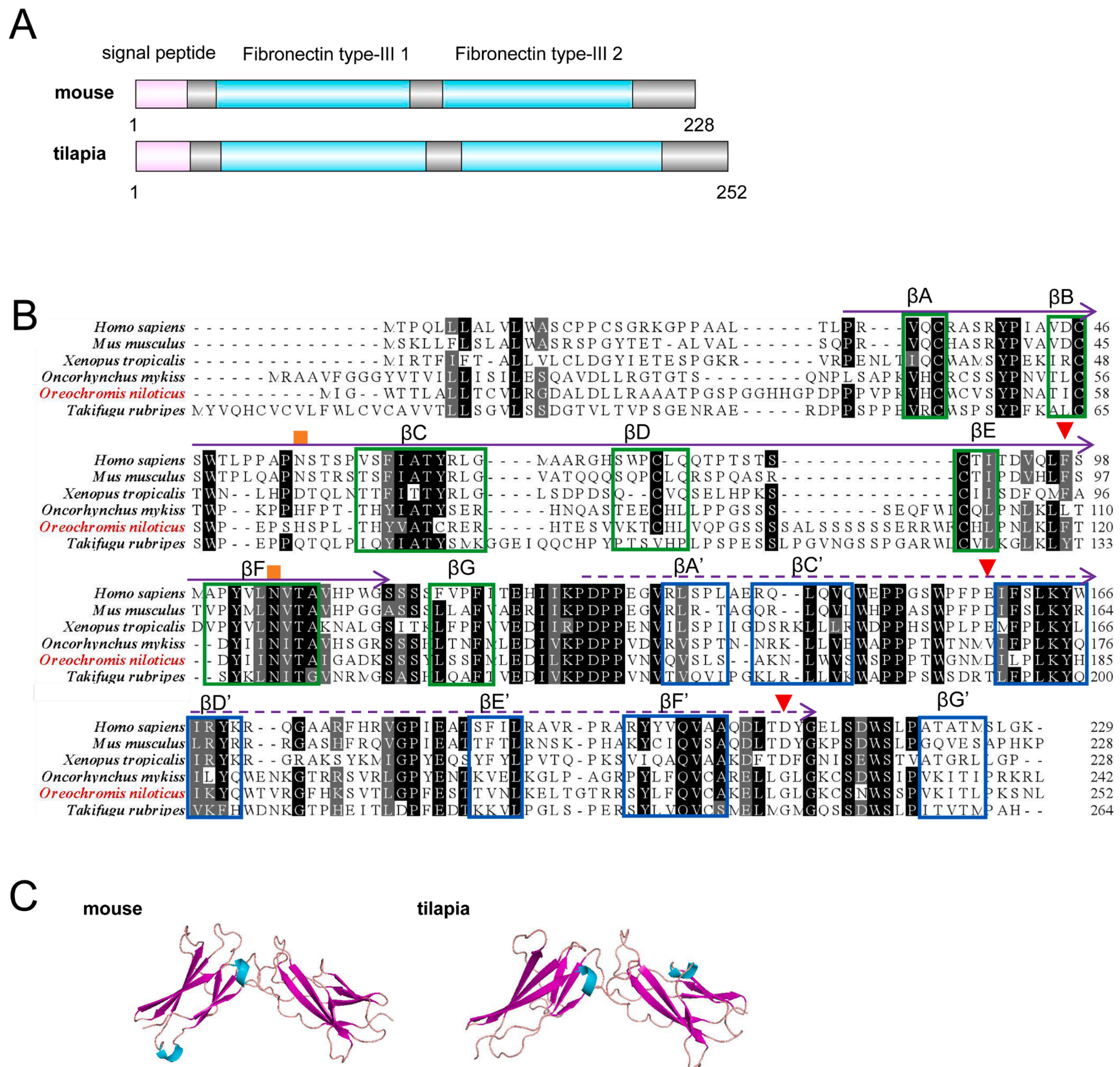

Fig. 2. Sequence analysis of IL-27β. (A) Predicted domains of IL-27β in tilapia and mouse. A signal peptide in pink, and a conserved FN III domain were shown. (B) Multiple sequence alignments of OnIL-27β and other homologues. Amino acid residues colored in black were conserved in 80% sequences and those colored in gray were similar residues. Some important regions and amino acid residues are specially labeled, with arrow region for FN III domain, blue box for β-stand, orange square for N-linked asparagine, and red triangle for key residue of IL-27 subunit action. (C) Predicted tertiary structures of IL-27β in tilapia and mouse.

subsequent experiments.

3.6. The ability of leukocytes to bind OnIL-27β

To further confirm the binding of IL-27β on tilapia leukocytes, head-kidney leukocytes were incubated with the recombinant IL-27β with His-tag, and then stained with anti-His and anti-CD3ε mAbs (Fig. 6). Head-kidney leukocytes are mainly composed of monocyte/macrophage (R1: ~15.9%), precursor cells (R2: ~28.6%), and lymphocytes (R3: ~26.6%) (Fig. 6A). The results showed that monocytes/macrophages can not bind rOnIL-27β, whereas ~57.2% of the precursor cells possessed the capability to bind to rOnIL-27β (Fig. 6B, C). ~80.9% of other lymphocytes (R4) and ~17.1% of T lymphocytes (R5) comprised the lymphocytes population (Fig. 6D), of which ~4.87% of other lymphocytes (R4) and ~9.47% of T lymphocytes (R5) were able to bind rOnIL-27β, respectively (Fig. 6E, F). This demonstrated that OnIL-27β had a wide range of target cell types and might have potential functions in the regulation of precursor cells and T cell-mediated immune responses.

3.7. OnIL-27β activates Erk and JNK pathways in lymphocytes

Studies have shown that cell growth, activation, differentiation, apoptosis and function are associated with the activation of Erk and JNK pathways [29]. To explore whether OnIL-27β was involved in the regulation of lymphocyte-mediated adaptive immune responses through these pathways, we stimulated spleen lymphocytes with rOnIL-27β and examined the changes in the phosphorylation levels of Erk1/2 and JNK, major components of the Erk and JNK pathways, respectively. Compared to the resting group, the phosphorylation levels of Erk1/2 and JNK increased rapidly and strongly at 15 min after treatment and decreased gradually at 30 min, and 4 h, implying that the involvement of OnIL-27 activated the Erk and JNK pathways (Fig. 7). Thus, the findings reveal the potential involvement of OnIL-27β in lymphocyte-mediated adaptive immune responses in tilapia by activating the Erk and JNK signaling pathways.

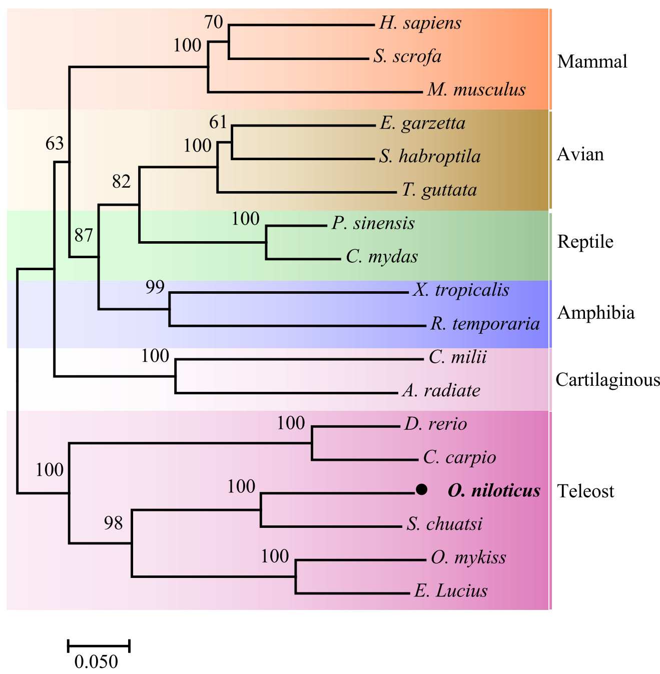

Fig. 3. Phylogenetic tree analysis of IL-27β. Evolutionary relationships were calculated based on the full-length protein sequences from vertebrates. The bootstrap values labeled at each node show the output from the NJ algorithm. The colored boxes show species of different evolutionary status. The information of the protein sequences is listed in Table S1.

3.8. OnIL-27β enhances Th1 responses by triggering the JAK1/STAT1/T-bet axis

Considering the importance of IL-27β in promoting Th1 cell activation, differentiation and function in mammals [18,23], we next sought to explore whether and how OnIL-27β is involved in the Th1 cell-mediated immune response in tilapia. The spleen lymphocytes of tilapia were treated with rOnIL-27β, and the expression of the Th1-critical cytokine IFN-γ was then examined at transcription levels. Administration of rOnIL-27β to spleen lymphocytes enhanced the transcript levels of IFN-γ (Fig. 8A). Next, we sought to determine whether this phenomenon was attributed to activation of the classical JAK/STAT pathway. The mRNA levels of JAK1 and STAT1 were significantly increased after rOnIL-27β treatment, instead of TYK2 and STAT4 (Fig. 8 B-E). Further, rOnIL-27β induced an elevated transcript level of the transcription factor T-bet, which is associated with Th1 differentiation (Fig. 8 F). Therefore, OnIL-27β may enhances Th1 cell activation and differentiation by activating the JAK1/STAT1/T-bet axis in tilapia.

4. Discussion

As a new member of the IL-12 family, IL-27 is a heterodimer cell factor composed of IL-27β and IL-27p28 [30]. IL-27 was initially identified as a proinflammatory cytokine, partly because it can promote NK cells to secrete IFN-γ, macrophages secrete IL-8 and promote Th1 cells to express IFN-γ [5]. However, studies in the past ten years have shown that IL-27 can antagonize the immune response of Th1, Th2, and Th17, and induce the differentiation of Treg and the secretion of IL-10 to limit the inflammatory response [31]. These findings showed that the immune function of IL-27 is not simply proinflammatory or anti-inflammatory, but determines its regulatory properties according to the immune microenvironment. Therefore, the pleiotropy and complexity of IL-27 endow it with the regulation in a variety of bacterial or viral infections, autoimmune diseases and tumors, and gradually determine it as a potential target for immunotherapy [32]. However,

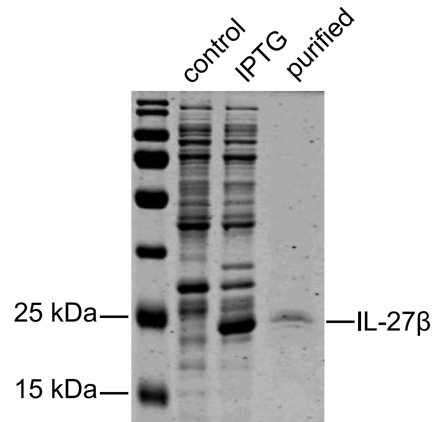

Fig. 5. Preparation of recombinant OnIL-27β. SDS-PAGE shown the recombinant protein expression with or without IPTG induction and purified of Nile tilapia IL-27β. The above experiments were repeated for at least two independent times.

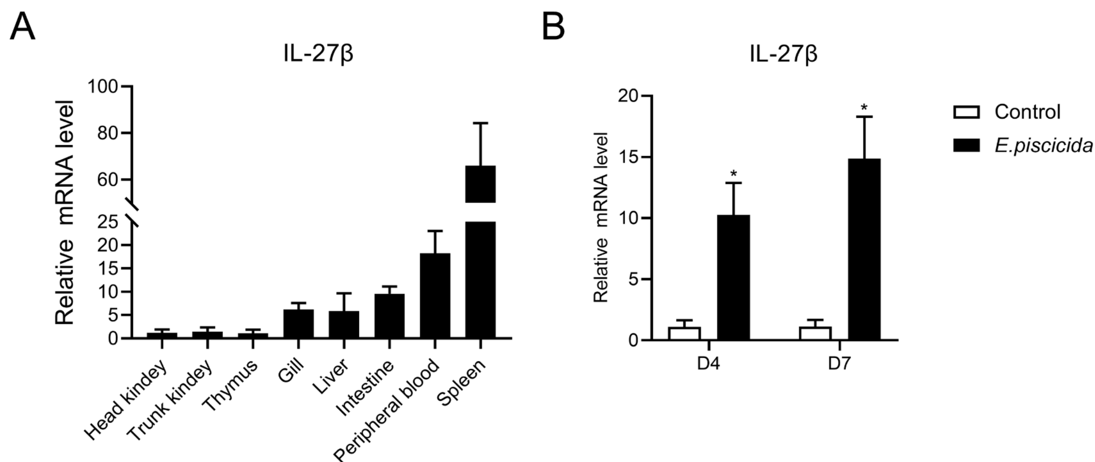

Fig. 4. Relative expression levels of IL-27β in Nile tilapia. (A) The tissue distribution of OnIL-27β detected by qPCR. The relative mRNA levels of OnIL-27β was shown as fold expression based upon spleen (n=4). (B) Relative mRNA levels of IL-27β in spleen lymphocytes at the indicated time point with or without *E. piscicida* infection were examined by qPCR (n=4). The above experiments were repeated for at least two independent times. The vertical bars represent mean ± SE, and the significance was shown (*: $P < 0.05$) as determined by a two-tailed Student's t-test.

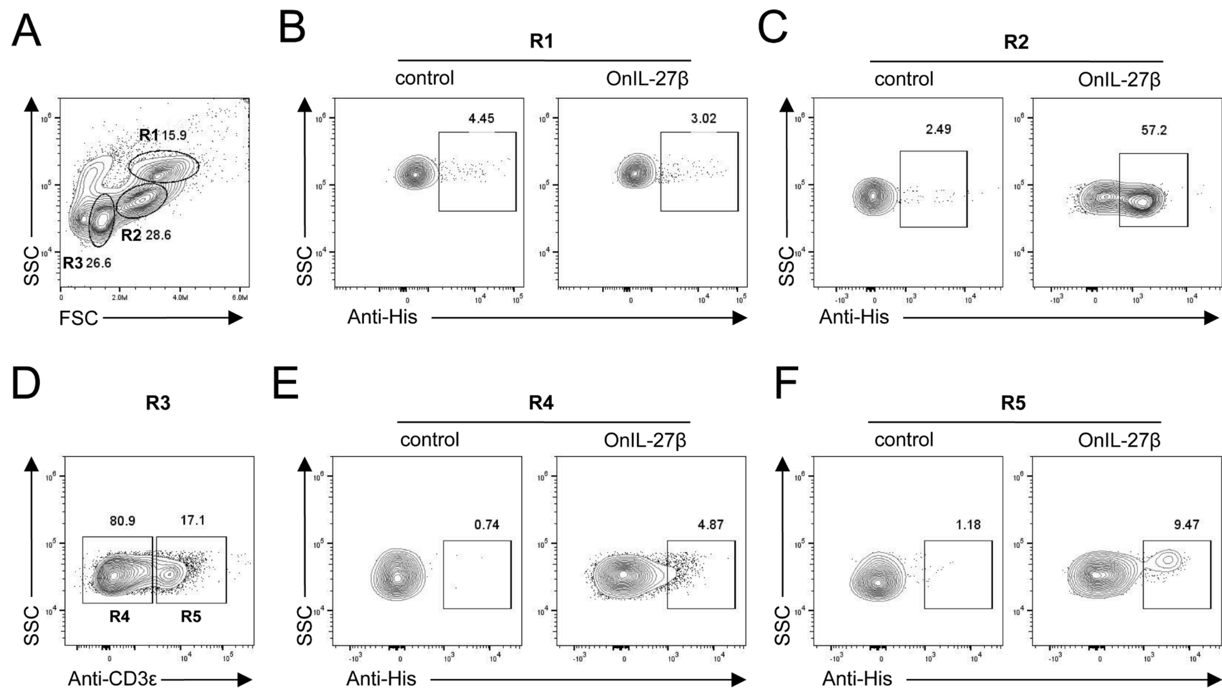

Fig. 6. Head-kidney leukocytes were incubated with recombinant His-tag-IL-27β, and then stained with the anti-His and anti-tilapia CD3ε mAbs. (A) Based on flow cytometry forward scatter (FSC) and side scatter analyses (SSC), three visible populations (R1-3) from head-kidney leukocytes were identified. (B) and (C) represented the ratio of leukocytes in R1 and R2 affected by OnIL-27β, respectively. (D) The ratio of head-kidney lymphocytes to T cells and other lymphocytes. (E) and (F) The ratio of IL-27β action to other lymphocytes and T lymphocytes in the head-kidney. In B, C, E, and F, the left: the control group without protein incubation, the right: the experimental group incubated with recombinant His-tag-IL-27β. R1: monocytes/macrophages, R2: precursor cells, R3: lymphocytes, R4: other lymphocytes, and R5: T lymphocytes.

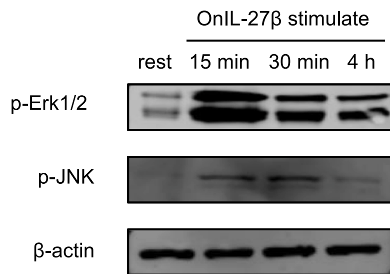

Fig. 7. Spleen lymphocytes were stimulated with 5 μg/mL rOnIL-27β for 15 min, 30 min, and 4 h, and phosphorylation level of Erk1/2 and JNK were examined by western blot.

IL-27p28 decreased the effect of IL-27β on Th17 and Treg, but did not affect Th1 cell differentiation [33]. This also provides a theoretical basis for the absence of IL-27p28 in tilapia. Although the presence and involvement of IL-27β in the immune response has been demonstrated in a variety of teleost, it is currently unclear how IL-27β is involved in the adaptive immune response. In this study, we identified an evolutionarily conserved IL-27β from Nile tilapia, and further revealed the involvement and potential mechanism of IL-27β in adaptive immune response in tilapia.

IL-27β is widely distributed in vertebrates. Collinear analysis showed that IL-27β of tilapia had obvious gene linkage with the upstream and downstream genes of IL-27β of humans, mouse, and pufferfish. The five exons of most of vertebrate IL-27βs are separated by four introns, but the gene structure of the multi-exon-intron has been maintained throughout evolution. Like other vertebrate IL-27βs, OnIL-27β also has the FN3 domain, which has been identified in a great deal proteins, mainly includes receptor protein tyrosine kinases, various cytokine related receptors and various proteins involved in cell surface binding [34]. And the FN3 domain is conserved in the IL-27β molecules of fish, amphibians, and mammals. Interestingly, in zebrafish and grass carp, the N-terminus of IL-27β has a unique Ig like domain, which is not contained in OnIL-27β [27]. Multiple sequence alignment revealed that tilapia IL-27β contained the β-stands and conserved functional motifs of vertebrates' IL-27βs. At the same time, these patterns of evolutionary protection for tilapia IL-27β were further confirmed by phylogenetic trees, and it is well clustered with other bony fish and tetrapod congeners. These observations suggested that tilapia IL-27β is evolutionarily conserved.

The predominant cellular source of IL-27 is thought to be the myeloid cell population, including dendritic cells, macrophages, monocytes, and microglia, in addition to some plasma cells and epithelial cells that also express IL-27 [4]. Studies in mammals have shown that IL-27 was widely distributed in various tissues, including tonsils, spleen, and liver, implying its involvement in the pathological response to inflammatory diseases in a variety of tissues [35–37]. Our studies showed that IL-27β was widely distributed in various immune-related tissues including spleen, peripheral blood, intestine, liver, and gill in tilapia, which provided a molecular basis for the rapid response of IL-27β to the immune response. Similarly, studies in grass carp also revealed extensive expression of IL-27β in a variety of immune-related tissues/organs, especially in blood, spleen, and head kidney [27]. It is worth noting that besides peripheral lymphoid tissue, we also observed IL-27β expressed in thymus, which indicates that IL-27β has a potential regulatory role in the regulation of lymphocyte development. A previous study found high expression of IL-27 in the blood of patients with systemic inflammatory response syndrome, therefore it was proposed as a candidate diagnostic biomarker for bacterial infections, suggesting the involvement of IL-27 in the body's immune response to pathogenic microorganisms [38]. In mice with TCCR/WSX-1 deletion, the reduced resistance to the intracellular pathogens, *Listeria monocytogenes* [38] and *Leishmania major* [32] suggested that IL-27 signaling facilitated resistance to pathogenic

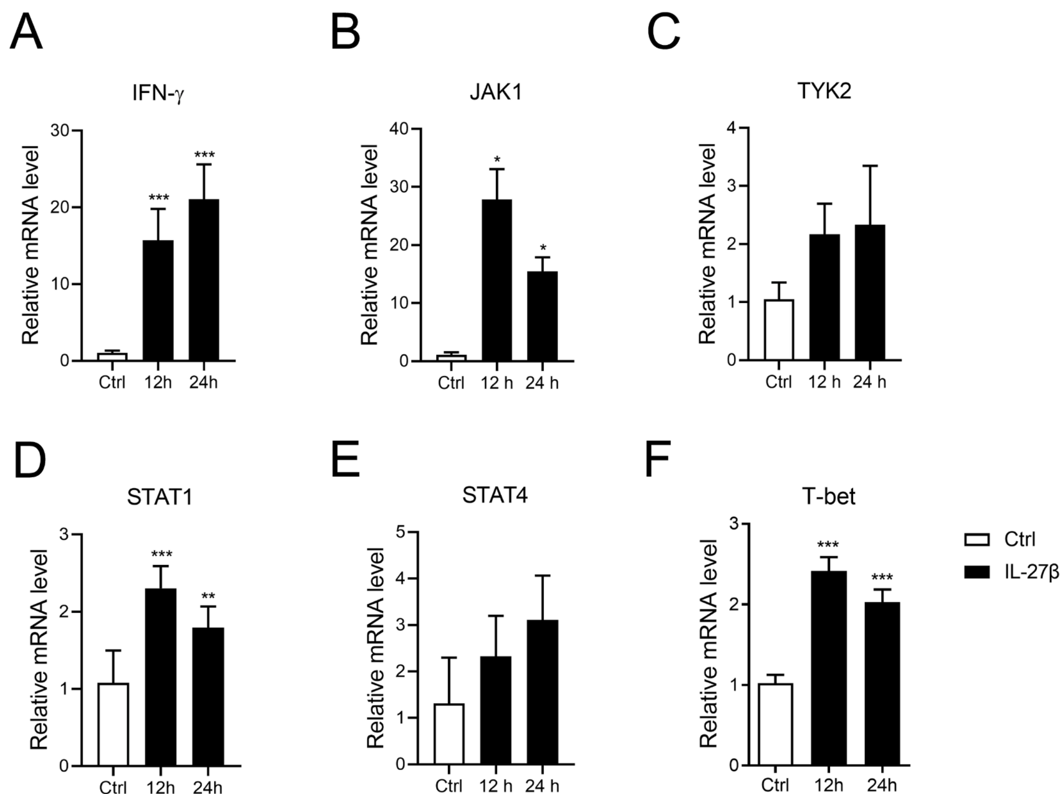

Fig. 8. Activation of downstream signaling pathway after IL-27β stimulation. IFN-γ (A), JAK1 (B), TYK2 (C), STAT1 (D), STAT4 (E), and T-bet (F) expression in Nile tilapia spleen lymphocytes after rIL-27β stimulation for 12 and 24 hours (n=4). The vertical bars represent mean ± SE, and the significance was shown (*: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$) as determined by a two-tailed Student's t-test.

invasion. However, data from WSX-1 deficient mice infected with *Toxoplasma gondii* [39] and *Trypanosoma cruzi* [40] indicated that the loss of IL-27 signal leads to enhanced cellular response. Overall, IL-27 has an important bidirectional regulatory role in anti-infective immunity, which may depend on the necessity of the immune microenvironment. However, With IL-27β was found in tongue sole, grass carp, and zebrafish, the expression of IL-27β in various tissues or lymphocytes can be induced by LPS, Poly I: C, *E. tarda*, and cytomegalovirus [26]. In this study, we demonstrated that OnIL-27β is significantly upregulated in a primary immune response induced by bacterial infection, which further suggests that IL-27β is involved in adaptive immune regulation in teleost.

IL-27 is a pleiotropic cytokine with multiple immunomodulatory activities under physiological and pathological conditions. Thus, IL-27 has a broad and pleiotropic modulatory effect on a wide range of immune cells, including innate immune cells and lymphocytes [41]. Studies in mammals have shown that IL-27 enhanced the cytotoxic activity of NK cells [42–44]; promoted the differentiation of monocytes into macrophages or DCs, and facilitated the release of pro-inflammatory cytokines and antiviral activity [42,45,46]. In addition, IL-27 accelerated the development, survival, and antibody secretion of B cells [47–49], and promoted Th1 cell differentiation and enhanced the development and function of $CD8^+$ T cells [32,50]. However, with the deepening of research, the anti-inflammatory effects of IL-27 are gradually being emphasized and recognized. For example, IL-27 inhibits the response of DCs, macrophages, Th1, Th2, and Th17 in a specific environment and limits the inflammatory response by inducing Treg differentiation and IL-10 secretion to avoid autoimmune diseases [39,51-56]. Further validation of the anti-inflammatory properties of IL-27 was obtained by the higher pro-inflammatory response induced by blocking IL-27 signaling during bacterial infection [31]. These studies demonstrate that the regulatory role of IL-27 on these immune cells is based on the needs of the immune microenvironment to determine whether the body requires greater pro-inflammatory activity to clear pathogens or to limit the inflammatory response to maintain immune homeostasis. Notably, our study demonstrated that OnIL-27β also had multiple types of potential target cell populations, including precursor cells, monocytes/macrophages, T cells and other lymphocytes (B cells, NK cells. etc). Our findings demonstrated the potential regulatory role of OnIL-27β act on multiple types of immune cells, however the specific direction and mechanism of regulation of these cells remains a goal for us to pursue in the future.

Although recent research showed that IL-27β upregulated the expression of the innate immune factors involved in T cell activation in tongue sole, there is no convincing evidence to show how this cytokine promotes adaptive immunity mediated by T cells. In our previous studies, Nile tilapia was used as a model to elucidate that several signaling pathways (namely Ca^{2+}-NFAT, MAPK/Erk, NF-κB, and mTORC1) jointly support activation, proliferation, and antimicrobial immune function of teleost T cells [28,57-59]. Advances in mammal research have established that T cell activation through via Erk and JNK pathways [60]. Based on the above studies, we found that rOnIL-27β acts extensively on lymphocytes, and it may regulate lymphocyte activation through the Erk or JNK signaling pathways. In addition, IL-27 has been found in mammals to induce Th1 differentiation through multiple signaling pathways [18]. It has been reported that IL-27 interacts with its receptors to recruit and activate JAK1 and TYK2, resulting in phosphorylation and dimerization of the transcription factor STAT1 or STAT3, which further forms dimers and is transported to the nucleus to promote the transcription of target genes, and promote Th1 cell activation and differentiation [61]. Simultaneously, activation of STAT1 promotes Th1 differentiation either through upregulating the signature cytokine of Th1 differentiation T-bet or via membrane ICAM-1, which interacts with LFA-1 and signals via Erk and JNK [62,63]. In general, all

of these T-bet activation pathways lead to IFN-γ secretion regulating Th1 cellular immunity. However, how IL-27β modulates downstream immune signaling remains unclear in teleost. Based on the above studies, we found that rOnIL-27β acts extensively on Th1, and it may enhance Th1 activation and differentiation through the conserved JAK1/STAT1/T-bet axis.

In conclusion, we identified an evolutionarily conserved cytokine IL-27β in Nile tilapia, which is involved in the bacterial-induced initial adaptive immune response. In addition, our study revealed potential regulatory target cell types of IL-27β in tilapia, and further studies identified the involvement of IL-27 in lymphocyte-mediated immune responses through activation of Erk and JNK signaling. More importantly, the enhanced activation and differentiation of Th1 cells in tilapia by IL-27β may be attributed partially to the activation of the JAK1/STAT1/T-bet axis. This study provides a theoretical basis for enriching the regulatory mechanisms of IL-27β in teleost and provides useful insights into the evolution of the adaptive immune system.

Declaration of Competing Interest

The authors have stated explicitly that there are no conflicts of interest in connection with this article.

Data availability

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

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.fsirep.2023.100087.

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