Sterile alpha and TIR motif-containing protein 1 is a negative regulator in the anti-bacterial immune responses in Nile tilapia (Oreochromis niloticus)

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Nile tilapia (Oreochromis niloticus) is one of the most important food fish in the world. However, the farming industry has encountered significant challenges, such as pathogen infections. Toll-like receptors (TLRs) play an essential role in the initiation of the innate immune system against pathogens. Sterile alpha and TIR motif-containing protein 1 (SARM1) is one of the most evolutionarily conserved TLR adaptors, and its orthologs are present in various species from worms to humans. SARM1 plays an important role in negatively regulating TLR domain-containing adaptor proteins inducing IFNβ (TRIF)-dependent TLR signaling in mammals, but its immune function remains poorly understood in fish. In this study, O. niloticus SARM1 (OnSARM1) was cloned and its evolutionary status was verified using bioinformatic analyses. mRNA expression of OnSARM1 was found at a higher level in the trunk kidney and muscle in healthy fish. The examination of its subcellular location showed that the OnSARM1 was detected only in the cytoplasm of THK cells, and colocalized with OnMyD88, OnTRIF and OnTRIF in small speckle-like condensed granules. The transcript levels of OnMyD88, OnTIRAP, OnTRIF, and downstream effectors, including interleukin (IL)-1β, IL-8, IL-12b and type I interferon (IFN)γd2.13, were regulated conversely to the expression of OnSARM1 in the head kidney from Aeromonas hydrophila and Streptococcus agalactiae infected fish. Moreover, the treatment of THK cells with lysates from A. hydrophila and S. agalactiae enhanced the activity of the NF-κB promoter, but the effects were inhibited in the OnSARM1 overexpressed THK cells. Overexpression of OnSARM1 alone did not activate the NF-κB-luciferase
Introduction

Aquaculture is a well-known activity that has spread globally. In 2020, the Food and Agriculture Organization (FAO) released statistical data showing that between 2001 and 2018, the global aquaculture production of farmed aquatic animals increased at a rate of 5.3% per year (1). Aquaculture will continue to be a leading source of global fish production, continuing a decades-long trend. The production of aquaculture is expected to reach 109 million tonnes in 2030, increasing by 32% (26 million tons) from 2018 (1).

Globally, tilapia is the second most important farmed fish, after carp. Despite the impact of COVID-19, the worldwide production of farmed tilapia increased by 3.3% in 2020, surpassing 6 million tons for the first time (2). Tilapia is a fast-growing fish that is relatively disease-resistant, yet several pathogenic microorganisms have jeopardized its production (3). The farming industry has encountered significant challenges from infectious pathogens, such as Streptococcus agalactiae, Streptococcus iniae, Aeromonas hydrophila, Edwardsiella tarda, and Flavobacterium spp. (4–7). Furthermore, the tilapia lake virus (TiLV) is a tilapia virus that causes mass deaths (8).

Innate immunity is an essential and widely distributed form of immunity representing the first line of defense against pathogens (9). Pathogens are identified by a variety of pattern-recognition receptors (PRRs) in the innate immune system, including toll-like receptors (TLRs), retinoic acid-inducible gene (RIG)-1-like receptors, the nucleotide-binding oligomerization domain (NOD)-like receptors, and C-type lectin receptors (10). The TLR family is one of the most important families in the PRRs, which recognize pathogens extracellularly or in intracellular organelles, such as endosomes and lysosomes (11).

Five TLR adaptor proteins have been recognized: the myeloid differentiation factor 88 (MyD88), the TIR domain-containing adaptor protein (TIRAP), the TIR domain-containing adaptor protein inducing IFNβ (TRIF), the TRIF-related adaptor molecules (TRAM), and the Sterile alpha and TIR motif-containing protein 1 (SARM1) (12). SARM1, the latest identified TLR adaptor protein (13), has been shown to be a negative regulator of TRIF in humans, in contrast to the other four adaptors that have shown activating effects (14). SARM1 is a highly conserved protein present in a variety of organisms, including the horseshoe crab (Limulus polyphemus), roundworm (Caenorhabditis elegans), amphioxus (Branchiostoma belcheri tsingtauense), zebrafish (Danio rerio), grass carp (Ctenopharyngodon idella), and whiteleg shrimp (Litopenaeus vannamei), revealing that it is ancient in its origins (13, 15–19).

SARM1 has been cloned from mammals and a few fish species and studies have uncovered its role as a negative regulator in antiviral pathways (13, 14, 20), yet its functions in antibacterial responses have never been properly scrutinized. To investigate this, we performed in vitro and in vivo bacterial stimulation/infection models and report that OnSARM1 negatively regulates NF-κB activity, thereby influencing the transcript level of proinflammatory cytokines and antimicrobial peptides.

Materials and methods

Experimental fish, infection, and sample collection

The Nile tilapia (Oreochromis niloticus) NT1 strain was generously provided by Prof. Hong-Yi Gong (Department of Aquaculture, National Taiwan Ocean University). The procedures for sampling tissues from healthy fish are described by Trung and Lee (21).

A total of 80 fish (average weight 45.8 ± 6.8 g) were acclimatized in four 90 L glass tanks (n = 20 each) that were aerated and supplied with dechlorinated tap water (28 ± 2°C). The photoperiod for each tank was maintained at 12 h light/12 h
dark. *A. hydrophila* and *Streptococcus agalactiae*, which had been isolated from diseased tilapia, were cultured on tryptic soy agar (TSA, Difco) at 28°C overnight. Cells were washed off with sterile phosphate-buffered saline (PBS, pH 7.4) and adjusted to the desired concentration. The experimental fish were weighed and injected intraperitoneally with PBS (control group), *A. hydrophila* (4 × 10⁵ colony forming units (CFU)/g of fish body weight), or *S. agalactiae* (1.3 × 10⁶ CFU/g of fish body weight) (n = 20 per group) (21). Tissues were collected (five fish per group) at 4, 8, 24, and 72 h post infection (hpi) and preserved in RNAlater solution (Invitrogen, CA, US). Fish handling and experiments were conducted following the guidelines of the Institutional Animal Care and Use Committee, which is approved by National Taiwan Ocean University (code: 109042).

Total RNA extraction, cDNA synthesis, and quantitative real-time polymerase chain reaction (qRT-PCR) assay

Total RNA was extracted using a TRI Reagent (Sigma-Aldrich, US) according to the manufacturers’ instructions. RNA quantity and purity were determined using a Spectrophotometer (Molecular Devices, US). Reverse transcription was conducted with 5 µg of total RNA using RevertAid™ reverse transcriptase (200 U/µl, Thermo Fisher Scientific, US). cDNA was diluted with 40 µl of RNase-free distilled water for gene cloning and stored at -20°C (21). The iScript™ gDNA Clear cDNA Synthesis Kit (BioRad, US) was used for cDNA synthesis for qRT-PCR analysis. The elongation factor-1α (EF-1α) (a reference gene) was used for normalization. Gene expression of *OnSARM1*, *OnMyD88*, *OnTIRAP*, *OnTRIF*, interleukin (IL)-1β, IL-8, IL-12a, IL-12b, tilapia piscidin (TP) 2, TP3, TP4, hepcidicine, tumor necrosis factor (TNF)-α, major histocompatibility complex class 1α (MHC Iα), and type 1 interferon (IFN)d2.8 and IFN2.13 were measured using a StepOnePlus Real-Time PCR instrument (Thermo Fisher Scientific) using the primers listed in Supplementary Table 1. The qRT-PCR reaction mixture (20 µl) was composed of 4 µl of cDNA template, 2 µl of specific primers, 4 µl of PCR-grade water, and 10 µl of RealQ Plus 2 × Master Mix Green (Ampliqon, Denmark). Reaction mixtures were incubated for 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, and finally 1 min at 60°C. A melting curve was generated by increasing the temperature from 60°C to 95°C with 0.3°C increments.

Cloning of *OnSARM1* and plasmid constructions

To clone Nile tilapia *SARM1*, a set of primers was designed based on the complete coding region of the *OnSARM1* sequence (Supplementary Table 1). The PCR reactions were conducted using MyTaq Red Mix 2× (Bioline, UK). The PCR thermal cycling was performed via an initial denaturation at 95°C for 1 min, followed by 35 cycles at 95°C for 15 s, 53°C for 30 s, and 72°C for 1.5 min, and a final extension at 72°C for 10 min. The obtained PCR product was cloned into the pGEM-T Easy Vector (Promega, US) and sequenced using Genomics Ltd.

The *OnSARM1*-GFP and *OnSARM1*-3×FLAG expression plasmids were prepared using primers GFP-Nhel-OnSARM1-F and GFP-HindIII-OnSARM1-R, and FLAG-HindIII-OnSARM1-FL-F and FLAG-XbaI-OnSARM1-FL-R, respectively (Supplementary Table 1). Q5® High-Fidelity DNA Polymerase (New England Biolabs, US) was used, and the PCR reactions were set up as follows: one cycle at 98°C for 30 s, followed by 35 cycles at 98°C for 15 s, 62°C for 30 s, 72°C for 90 s, and terminated with one cycle of 72°C for 10 min and 95°C for 60 s. The purified PCR products and empty pTurbo-GFP-N (Evrogen, Russia) or p3×FLAG-CMV-14 vector (Sigma-Aldrich) were ligated together after restriction enzyme digestion. Ligation products were transformed into ECOS™ 101 Competent Cells (DH5α™) (Yeastern Biotech, Taiwan). Positive colonies were screened using vector-specific primers (Supplementary Table 1), and colonies with correct insert size were grown in LB broth with ampicillin (100 µg/ml) (for p3×FLAG-CMV-14 vector) or kanamycin (50 µg/ml) (for pTurboGFP-N vector) for plasmid preparation.

Bioinformatics

Homologous sequences were searched through the BLAST algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The nucleotide sequence of *OnSARM1* was translated using the ExPaSy translate tool (http://web.expasy.org/translate/). The molecular weight of the protein was calculated using the Compute pI/Mw tool (http://web.expasy.org/compute_pi/). A phylogenetic tree was constructed using the neighbor-joining method in Molecular Evolution Genetics Analysis software version 7 (MEGA7), supported by 10,000 bootstrap repetitions with the Poisson model for amino acid substitution and pairwise deletion for gap treatment (22). EMBOSS Needle online software (https://www.ebi.ac.uk/Tools/psa/emboss_needle/) was used to identify the sequence identity and similarity between vertebrates *OnSARM1* (23). Synteny analysis was performed via the Genomics v100.01 program (24). Simple Modular Architecture Research Tool (SMART) (http://smart.embl-heidelberg.de/) was used to predict the deduced amino acid sequence of *OnSARM1* (25). Multiple alignments were performed using the ClustalW program (https://www.ebi.ac.uk/Tools/msa/clustalw/).

Cell culture

A tilapia head kidney (THK) cell line (26) was cultured at 27°C in Leibovitz medium (L-15, Gibco) containing 10% Fetal
Bovine Serum (FBS, Corning) and 1% penicillin (100 U/ml)/streptomycin (100 μg/ml) (P/S, Gibco). Human embryonic kidney (HEK) 293 cells were provided by Prof. Pin-Wen Chiou (Department of Aquaculture, National Taiwan Ocean University) and were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM, GE Healthcare Life Sciences) containing 10% FBS and 1% P/S at 37°C with 5% CO2 in a humidified incubator. When the cells reached 70-80% confluency, they were passaged or used for seeding.

### Cellular localization of OnSARM1

HEK 293 and THK cells (1 × 10^5 cells per well) were seeded onto glass coverslips in 24-well culture plates with 1 ml culture medium 16 h before transfection. The following day, 1,000 ng of pTurbo-OnSARM1-GFP plasmids in serum-free medium was mixed with TurboFect Transfection Reagent (Thermo Fisher Scientific) and incubated for 20 min at room temperature following the manufacturer’s instructions before being added to the cells. Forty-eight hours after transfection, the cells were washed thrice with 500 μl of Hank’s Balanced Salt Solution (HBSS; Thermo Fisher Scientific) and fixed with 500 μl of 4% paraformaldehyde (PFA) at room temperature for 15 min. Subsequently, cells were washed thrice with HBSS and incubated for 30 min with HBSS containing Alexa Fluor 546 phalloidin (Thermo Fisher Scientific) in a darkened room. After cells were washed thrice with HBSS again, cells fixed on the coverslip were stained with VECTASHIELD Antifade Mounting Medium containing DAPI to visualize the nucleus (blue). The coverslip was sealed with nail polish. Finally, the sealed coverslips were observed using a confocal microscope (Zeiss LSM 880 with Airyscan). The colocalization efficiency was quantified by observing the overlap of the fluorescence intensity peaks along the white line by line scan analysis profiles (ZEISS ZEN 2.6 (blue edition) software).

### Western blotting

Western blotting was conducted according to procedures described in previous research (28). HEK 293 cells were seeded in 24-well plates at a density of 1 × 10^5 cells per well the day before transfection. The TurboFect Transfection Reagent was used to transfect cells with the indicated plasmids or left untreated as a negative control. Plasmids encoding Nile tilapia MyD88, TRIF, and TIRAP were prepared previously (21, 27). Cell lysates from transfected cells were separated by gradient SDS-PAGE (Bionovas, Canada) before being transferred to a PVDF membrane (Millipore, US). The membrane was immunoblotted with the anti-TurboGFP (d) antibody (1:1000) (Evrogen) or ANTI-FLAG M2 antibody (1:1000) (Sigma-Aldrich). The membrane was then incubated with peroxidase-conjugated AffiniPure goat anti-rabbit or goat anti-mouse IgG (H+L) antibodies (1:5000, Jackson ImmunoResearch Laboratories, US). LuminolPen (Visual Protein) was used to mark the pre-stained molecular weight standard markers, and a SuperLight Chemiluminescent HRP Kit (Bionovas) was used for protein detection. The membrane was visualized using the UVP GelStudio PLUS Touch Imaging System (Analytik Jena, Germany).

### Luciferase assay

To investigate the luciferase activity induced by OnMyD88, OnTRIF, OnTIRAP, and OnSARM1 expressing plasmids, THK cells (5 × 10^4 cells per well) were seeded in 48-well culture plates with 500 μl of culture medium 16 h before transfection. The cells were then transfected with 250 ng of each expression plasmid or a corresponding empty vector (pcDNA3.0-HA for OnMyD88, OnTRIF, and OnTIRAP and p3×FLAG-CMV-14 vector for OnSARM1), with 250 ng of pNF-B-Luc (Clontech). Forty-eight hours post transfection, luciferase activity was determined using the luciferase reporter assay system (GeneCopoeia, US) according to the manufacturer’s instructions. Each experiment was performed in triplicate.

To verify the influence of OnSARM1 on the NF-kB activity mediated by OnMyD88, OnTRIF, and OnTIRAP, THK cells were transfected with 125 ng of OnMyD88, OnTRIF, or
Gene expression analyses in OnSARM1 overexpressed THK cells post A. hydrophila stimulation

THK cells (2.5 × 10^5 cells per well in 12 well plate) were seeded the night before transfection. The next day, the cells were transfected with an empty vector (2000 ng per well) or OnSARM1-3×FLAG expression plasmids mixed with an empty vector (1000 ng each). The cells were then stimulated for 4 h with lysates from A. hydrophila (10 μg protein/ml culture medium) and S. agalactiae (10 μg protein/ml culture medium) for 16 h, followed by luciferase assay analysis.

Results

Statistical analysis

SPSS 25 software (IBM, US) was used to analyze all quantitative real-time PCR data. The expression levels of the OnSARM1 were first normalized to the housekeeping gene EF-1α, and log2 transformed (29). An unpaired-sample t-test was performed to analyze the significant differences between the treatment and control groups in gene expression levels at each time point. One-way ANOVA (analysis of variance) and Tukey’s test were conducted to analyze the differences in each group in the luciferase assay. P-value < 0.05 was considered significantly different.

Expression of OnSARM1 in tissues of healthy fish

The gene expression of OnSARM1 in the tissues from healthy Nile tilapia is shown in Figure 5. The expression of OnSARM1 was highly expressed in the trunk kidney (64.2-fold) followed by the muscle (54.1-fold), when compared with the liver that exhibited the lowest expression (set as 1), and it was expressed moderately in the intestine, spleen, gills, skin, and head kidney.
Cellular localization of OnSARM1

An expression plasmid (pTurbo-OnSARM1-GFP), which encoded for OnSARM1 tagged with GFP at the C-terminus of the TIR domain, was constructed to study the cellular location of OnSARM1. Successful expression of the fusion protein was confirmed by the Western blotting of lysates from pTurbo-OnSARM1-GFP transfected HEK 293 and THK cells (Supplementary Figure 2). The molecular weight of the OnSARM1-GFP fusion protein was approximately 106 kDa (26 kDa Turbo-GFP plus 80 kDa for SARM1), while the pTurbo-GFP vector transfected cells produced a 26 kDa GFP protein. OnSARM1-GFP fusion protein was detected only in the cytoplasm of HEK 293 and THK cells (Figure 6).

Gene expression analysis in Nile tilapia after being challenged with S. agalactiae and A. hydrophila

The expression profiles of OnSARM1, OnMyD88, OnTIRAP, OnTRIF, IL-1β, IL-8, IL-12b, IFNβ2.8 and IFNβ2.13 were determined in the head kidney after a challenge with Gram-positive bacteria (S. agalactiae) (Figure 7). The expression levels of OnSARM1 (Figure 7A) and IFNβ2.8 (Figure 7H) were not significantly altered at any of the time points examined, while the transcript levels of OnTIRAP (Figure 7C) and OnTRIF (Figure 7D) increased significantly in the head kidney at 3 and 4 time points after being challenged, and OnMyD88 was upregulated at 24 h (Figure 7B). Significant upregulation of IL-1β (Figure 7E) and IL-8 (Figure 7F) was observed at all of the time points examined post S. agalactiae infection. Elevated gene expression of IL-12b and IFNβ2.13 was noticed at 8 h and 72 h, and 4 h and 24 h, respectively in the head kidney tissue from the S. agalactiae challenged fish.

In the fish challenged with Gram-negative bacteria (A. hydrophila), the transcript levels of OnSARM1 (Figure 8A) were promoted at 24 h and 72 h, while OnMyD88 was downregulated in the head kidney after the challenge injection (Figure 7B). The expressions of OnTIRAP (Figure 8C) and OnTRIF (Figure 8D) were upregulated at 4 and 3 time points post A. hydrophila infection. Similarly, the expression of IL-1β (Figure 8E) and IL-8 (Figure 8F) reached the highest level at 8 h, and decreased gradually at later time points. The expression of IL-12b (Figure 8G) and IFNβ2.8 (Figure 8H) remained almost at the basal level, but IFNβ2.13 (Figure 8I) was upregulated at 4 h and 8 h in the head kidney post A. hydrophila infection.

Interplay of OnMyD88, OnTRIF, OnTIRAP and OnSARM1 and their influence in NF-κB activation

To further study the regulatory role of OnSARM1 in immune cells, we adopted a cell line derived from tilapia head...
kidney with characteristics of melanomacrophages (26). Enhanced NF-κB promoter activity was seen in the empty vector transfected THK cells after treatment of the lysates from *A. hydrophila* (8.17 ± 2.05 fold) and *S. agalactiae* (2.42 ± 0.16 fold). Interestingly, *A. hydrophila*-mediated induction of NF-κB promoter activity was significantly suppressed in the OnSARM1 overexpressed THK cells (1.93 ± 0.21 fold) (Figure 9A). Although insignificant, this inhibitory effect was also seen in the *S. agalactiae* treated THK cells that overexpressed OnSARM1 (1.99 ± 0.31 fold).

In order to investigate the interaction among OnSARM1 and other TLR adaptors, the corresponding expressing plasmids were transfected into THK cells in combination with NF-κB reporter plasmids. As shown in Figure 9B, overexpression of OnMyD88 and OnTIRAP alone, but not OnTRIF and OnSARM1, activated NF-κB activity in THK cells, but the co-expression of OnSARM1 with three other adaptors, namely OnMyD88, OnTRIF and OnTIRAP, impaired the activation of the NF-κB reporter (Figures 9C–E).

### Co-localization of OnSARM1 and other TLR adaptors

Studies have shown that SARM1 could physically interact with other TLR adaptors by using co-immunoprecipitation and Western blotting (20), but whether OnSARM1 could co-localize with other TLR adaptors still needs to be determined. To this end, THK cells were transfected with OnSARM1-3×FLAG together with HA-tagged OnTRIF, OnTIRAP or OnMyD88 expression plasmids respectively, and the cells were stained with corresponding antibodies 48 h post-transfection. As shown in Figure 10, OnSARM1 were detected not only scattered in the cytoplasmic...
FIGURE 3
Multiple alignments of SARM1 amino acid sequences from Oreochromis niloticus, Homo sapiens, Mus musculus, and Ctenopharyngodon idella. The accession numbers of the amino acid sequences are provided in Supplementary Table 2. The dashes in the sequences indicate gaps introduced to maximize alignment. Identical (*), similar (.), and highly conserved (:) residues identified by the ClustalW program are indicated. Five domains are labeled and shaded in grey. ARM, Armadillo/beta-catenin-like repeats; SAM, Sterile alpha motif; and TIR, Toll - interleukin 1 - receptor.

FIGURE 4
Gene synteny analysis of segments containing SARM1 from different species. Gene order on the chromosome of each species was collected using the Genomicus database (Version 100.01). Gene distance was not drawn to scale. Gene names are given as follows: CHCHD2, Coiled-coil-helix-coiled-coil-helix domain containing 2; FAM98B, Family With Sequence Similarity 98 Member B; PRPF8, Pre-mRNA-processing-splicing factor B; RILP, Rab-interacting lysosomal protein; SCARF1, Scavenger Receptor Class F Member 1; VTN, vitronectin; SARM1, Sterile alpha and TIR motif-containing protein 1; SLC46A1, Solute Carrier Family 46 Member 1; FOXN1, Forkhead box protein N1; UNC119A, Protein unc-119 homolog A; CRYBB1L3, crystallin beta B1l; CRYBA1A, Beta A1-crystalline; C22orf31, chromosome 22, open reading frame 31; CPDA, 3',5'-cyclic adenosine monophosphate phosphodiesterase; GOSR1, Golgi SNAP receptor complex member 1; TRARG1, Trafficking regulator of GLUT4 1; AIPL1, Aryl-hydrocarbon-interacting protein-like 1; UNC119, Uncordinated-119; VTN, Vitronectin; CRYBA1, Crystallin Beta A1; and CPD, Carboxypeptidase D.
region but also formed in discrete foci in the cytosolic region that were found to be partially co-localized with OnTRIF (Figure 10A) or OnMyD88 (Figure 10C), which are present as small speckle-like condensed granules in plasmid transfected THK cells. OnTIRAP was found to be more evenly distributed in cytosol and co-localized with OnSARM1 (Figure 10B). Colocalization of OnSARM1 with the three TLR adaptors was visible as yellow coloring in the merged images and was further confirmed by the correspondence of the peak fluorescence intensities of the indicated proteins in the line scan graphs (Figure 10, bottom row).

OnSARM1 inhibits proinflammatory cytokine and antimicrobial peptide genes in THK cells post *A. hydrophila* lysate stimulation

We next examined the effect of OnSARM1 on anti-*A. hydrophila* dependent gene induction in more detail by comparing the mRNA expression level of immune genes in empty vector or OnSARM1 expressing plasmid transfected THK cells after stimulation with lysate from *A. hydrophila*.
FIGURE 7
Gene expression of (A) SARM1, (B) MyD88, (C) TRAP, (D) TRIF, (E) IL-1β, (F) IL-8, (G) IL-12b, (H) IFNα2.8, and (I) IFNα2.13 in the head kidney from Oreochromis niloticus challenged with Streptococcus agalactiae. Expression of the indicated genes was normalized to the expression level of EF-1α and expressed as fold change relative to the control group. The values are shown as mean ± SD (n = 3). Significant differences from the control group at each time point are indicated by asterisks (P < 0.05, unpaired sample t-test).

FIGURE 8
Gene expression of (A) SARM1, (B) MyD88, (C) TRAP, (D) TRIF, (E) IL-1β, (F) IL-8, (G) IL-12b, (H) IFNα2.8, and (I) IFNα2.13 in the head kidney from Oreochromis niloticus challenged with Aeromonas hydrophila. Expression of the indicated genes was normalized to the expression level of EF-1α and expressed as fold change relative to the control group. The values are shown as mean ± SD (n = 3). Significant differences from the control group at each time point are indicated by asterisks (P < 0.05, unpaired sample t-test).
As shown in Figure 11, expression of MyD88, TRIF and TIRAP (TLR adaptors), tilapia piscidin 2 (TP2) and TP3 (antimicrobial peptide), major histocompatibility complex class Ia (MHC Ia), interferon (IFN)α2.8 and IFNβ2.13, and IL-10 remained at a similar level compared to the empty vector transfected group. In contrast, hepcidin (antimicrobial peptide) was upregulated in the empty vector group to nearly 14-fold (13.94 ± 3.89 fold) after stimulation with lysate from Aeromonas hydrophila (Ah, 10 µg bacterial protein/ml culture medium) or Streptococcus agalactiae (Sa, 10 µg bacterial protein/ml culture medium) for 16 h; this was followed by luciferase assay analysis (n = 3). The values are shown as mean ± SD. Significant differences between the OnSARM1 or vector transfected group ([A], unpaired sample t-test) or between the experimental groups ([B–E], one-way ANOVA and Tukey's post-hoc test) are indicated by asterisks or different letters (P < 0.05), respectively.

### Discussion

Among the five known TLR adaptors, MyD88, TIRAP, TRIF, TRAM (absent in the fish genomes), and SARM1, SARM1 was the last to be identified. It is a pro-apoptotic protein in mammals that functions as a suppressor of TLR signaling (14, 30). However, in teleosts, SARM1’s function and role remain unclear. In this study, the CDS of OnSARM1 was cloned, and bioinformatic analyses suggested that the cloned gene was a homolog of SARM1 in Nile tilapia.

The results of the sequence alignment showed that OnSARM1 contained two N-terminal ARM domains, two central SAM motifs, and a C-terminal TIR domain, which is identical to the homologs from grass carp, mouse, and humans (13). In mammals, the first 27 amino acids at the N-terminal of SARM have been shown to be hydrophobic and polybasic, and to act as a mitochondria-targeting signal sequence for associating SARM to the mitochondria (30). From the sequence alignment, we noticed that four amino acids “GPRP” out of the first 27 amino acids of SARM1 were missing in fish, but the specificity of the mitochondria-targeting ability of the arginine residue at the 14th position was conserved between fish and mammalian SARM1 (Figure 3), suggesting that the subcellular localization of fish SARM1 is a conserved feature. Indeed, CiSARM1 was identified as specifically localized to mitochondria in the CIK cell (13).

The basal expression patterns of SARM1 were found to differ among various species. For example, CiSARM1 was expressed at high levels in the foregut, skin, and eye and at a low level in the midgut (13), while the homologous gene was expressed at high levels in the heart and gills in Litopenaeus vannamei and at a low level in the hemocyte (18). In mandarin fish (Siniperca chuatsi), SARM1 was detected in all of the tissues examined and was found to be highly expressed in the trunk kidney, skin, intestine, and pyloric caecum (20). In this study, the expression of OnSARM1 was found to be at a high level in the trunk kidney and muscle, and its lowest expression was in the liver.
The head kidney is an important lymphoid tissue in the immune system of fish (31). So far, only a few investigations have studied SARM1 expression modulation in fish challenged with bacteria. We therefore investigated the expression of OnSARM1 in the head kidney after challenging Nile tilapia with two pathogenic bacteria, *A. hydrophila* and *S. agalactiae*. The expression of OnMyD88 was not always modulated in the same way as OnTIRAP, and we also noticed that the expression of IL-1β was modulated in a similar way to that of OnTIRAP and OnTRIF, but was not correlated with the regulation of OnMyD88 in the head kidney from bacterial challenged fish. IL-1β is a critical pro-inflammatory cytokine that is essential in response to infection.
and is thought to be induced mainly through the TLR/MyD88/NF-κB signaling pathway in monocytes/macrophages (32). However, a recent study has elegantly demonstrated that both MyD88 and TRIF contribute to the priming of IL-1β expression and pro–IL-1β protein production in murine myeloid DCs after monophosphoryl lipid A (MLA, a derivative of LPS) stimulation (33). Additionally, TIRAP, but not MyD88, was shown to play an essential role in phosphoinositide 3-kinase (PI3K) activity and NF-κB activation upon diacylated lipoprotein stimulation (34). Therefore, a plausible explanation of the induction of IL-1ß (and other proinflammatory cytokines) while MyD88 was downregulated (or not upregulated) in the head kidney from A. hydrophila and S. agalactiae injected fish is that it is likely to be triggered by the TRIF- and/or TIRAP-dependent pathways.

IL-8, a CXC chemokine, is produced by macrophages and other cell types to mainly attract and activate neutrophils (35), and is regulated through the TLR/MyD88/NF-κB signaling pathway (36, 37). Studies have revealed that the activation of MyD88-dependent signaling pathways will promote the expression of IL-1ß and IL-12b (p40) in murine peritoneal macrophages and RAW264.7 cells (38, 39) and IL-8 in porcine alveolar macrophages (37). Herein, the transcript level of OnIL-8 was sharply induced by bacterial infection/stimulation (for THK cells), indicating that it is a significant factor in host defense in the head kidney and in the pathogenesis of bacterial infection in Nile tilapia.

IL-12 is a heterodimeric cytokine composed of the p35 (known as IL-12a) and p40 (known as IL-12b) subunits, which are important for stimulating the development of T helper 1 cells and cell-mediated immunity against pathogens (40). IL-12 is secreted by a range of cell types, including macrophages, neutrophils, and dendritic cells. Various microbial specific signatures (e.g., LPS, CpG, and peptidoglycan) are recognized by TLRs (41), leading to induction of the expression of IL-12b in the MyD88/TIRAP-dependent pathway (42), which is (partially) regulated by NF-κB (p50/p65 and p50/c-Rel) complexes in phagocytes (40). The expression of OnIL-12b was greatly enhanced in the head kidney after S. agalactiae infection
(moderately upregulated by *A. hydrophila* infection) and in the THK cells by the treatment of *A. hydrophila* lysate, possibly due to the activated MyD88/TIRAP-NF-κB-IL-12b cascade. Interestingly, *OnIL-12b* expression was inhibited in the OnSARM1 overexpressed THK cells; such a suppressive effect was also seen in adult brains from SARM1 knockdown transgenic mice (43).

Type I IFNs can be induced by viruses and bacteria. The main producing cells are macrophages, dendritic cells, and other hemopoietic cells (44). Stimulation of murine bone-marrow-derived DCs with *Lactobacillus acidophilus* (45) or murine macrophages with lipoteichoic acid (LTA) from *Staphylococcus aureus* (46) induced IFN-β or IFN-α production, respectively, in a TLR2-dependent pathway. Additionally, unmethylated CpG DNA, which is expressed approximately 20 times more frequently in bacterial than vertebrate DNA (47), was recognized by TLR9 (48). TRIF is a signaling adaptor, which can activate IRF3, NF-κB, and API and induce type I IFN and cytokines (49, 50). In this study, the expression of OnSARM1 and OnTRIF was mutually fluctuated in the immune tissues after *A. hydrophila* was administered, signifying that OnSARM1 and OnTRIF may act together in the antimicrobial immune responses but are regulated in the opposite way. For example, when the fish were infected with *A. hydrophila*, the transcript levels of OnTRIF and OnIFNαd2.13 were upregulated in the head kidney at early time points (4 h and 8 h), while the OnSARM1 expression was unaltered; however, OnSARM1 was upregulated at later time points (24 h and 72 h), whereas the transcript levels of OnTRIF and OnIFNαd2.13 reverted to a level similar to that of the control group (Figure 8). This suggests that OnSARM1 plays a role as a negative regulator in the TRIF-mediated pathway to prevent excessive inflammation (14).

Bacterial infection (or lysates) causes the delivery of ligands, such as LTA, peptidoglycan, flagellin, single-stranded (ss) RNA, double-stranded (ds) RNA and CpG DNA, to the host, which activates multiple TLRs, such as TLR1-3, TLR5, TLR7-9, TLR21, and teleost-specific TLR19 (51) and TLR22 (52). Additionally, our recent research revealed that the transcript levels of Nile tilapia TLR18 (27) and TLR25 (28) were elevated in the head kidney during bacterial infection and that these two fish-specific TLRs can physically interact with both MyD88 and TRIF, adding the possibility that they may recognize (unknown) bacteria-derived ligands and launch the production of proinflammatory cytokines and type I IFNs after activation.

Subsequent to an investigation conducted on human SARM (14), studies have also suggested that fish SARM serves as a negative regulator in antiviral responses (13, 20). However, the function of SARM1 in antibacterial responses at the cellular level remains unexplored in teleost. Here, the NF-κB reporter was activated in the *A. hydrophila* and *S. agalactiae* treated THK cells, and for the first time we showed that this inductive effect was inhibited in the OnSARM1 overexpressed THK cells (Figure 9). Moreover, gene expression of proinflammatory cytokines and hepcidin was evoked in THK cells by the treatment of lysate derived from *A. hydrophila*, but was suppressed when OnSARM1 was overexpressed (Figure 11), supporting the notion that SARM1 acts as a negative regulator in antibacterial responses in fish.

Carty et al. demonstrated that human MyD88- and TIRAP-mediated NF-κB activation in HEK 293 cells was unaffected by SARM expression, while TRIF-induced NF-κB activity was inhibited by SARM1 in a dose-dependent manner (14). By contrast, co-expression of mandarin fish MyD88, TRIF, or TIRAP with SARM1 in HEK 293 cells resulted in impaired NF-κB promoter activity mediated by the former three adaptors (20). Overexpression of OnMyD88 and OnTIRAP can induce the activity of the NF-κB-luc reporter in HEK 293 cells (21, 27) and THK cells, indicating that the function of these TLR adaptors in fish is conserved, as found in mammals. Similar to the findings in mandarin fish, overexpression of OnSARM1 suppressed OnMyD88- and OnTIRAP-mediated NF-κB promoter activity, presumably through the physical interaction of the BB-loop of the SARM1 TIR domain and the TIR domain from MyD88 and TIRAP (53). Additionally, Carlsson et al. showed that both SARM and MyD88 localize to the mitochondria (53), which is similar to the results from the confocal microscopy analysis in the present study, in which OnSARM1 colocalized with OnTRIF, OnMyD88 or OnTIRAP in small speckle-like condensed granules in the cytoplasm. Although enhanced NF-κB activity was seen in OnTRIF overexpressed HEK 293 cells (27), this was not the case when transfecting the same expression plasmid (for overexpressing OnTRIF) in the THK cells, and the presence of OnSARM1 had no influence on the activity of the NF-κB-luc reporter. The underlying mechanisms for the discrepancy in the OnTRIF-mediated NF-κB activity between human and fish cells need to be identified and may be of interest for further research.

**Conclusions**

In conclusion, OnSARM1 was cloned, identified, and characterized in Nile tilapia. The expression of OnSARM1 and other TLR adaptors was regulated in the head kidney after microbial stimulation. OnSARM1 colocalized with OnMyD88, OnTIRAP or OnTRIF in the cytoplasm of cells. Moreover, OnSARM1 alone was unable to activate the NF-κB reporter, but can suppress NF-κB promoter activity mediated by OnMyD88, OnTRIF and OnTIRAP in THK cells. Finally, OnSARM1 is likely to serve as a negative regulator in the NF-κB pathway during antibacterial responses, and inhibit the gene expression of proinflammatory cytokines and hepcidin in melanomacrophage-like cells.

**Data availability statement**

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.
Ethics statement

The animal study was reviewed and approved by NTOU Institutional Animal Care and Use Committee (approval number: 109042).

Author contributions

P-TL designed and supervised the study, PN, NT, H-YH, J-YL, EW, and AW conducted the study and data analysis. PN and NT wrote the first version of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.940877/full#supplementary-material
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