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Suppressor of cytokine signaling 3 (SOCS3) is related to pro-inflammatory cytokine production and triglyceride deposition in turbot (*Scophthalmus maximus*)

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ABSTRACT

Turbot (*Scophthalmus maximus*) is an economically important fish that is farmed by aquaculture for human consumption. Aquacultured turbot are commonly fed a high-lipid diet; however, this diet causes excessive lipid deposition and the overexpression of pro-inflammatory cytokines. Studies in mammals have indicated that a relationship exists between pro-inflammatory cytokine overexpression and altered lipid metabolism through the activation of suppressor of cytokine signaling 3 (SOCS3). In this study, we investigated the relationship between SOCS3 and triglyceride (TG) deposition and mechanism of SOCS3 activation in farmed turbot fed high-lipid diet (HLD). TG content increased with SOCS3 production, mediated by toll-like receptor-nuclear transcription factor kappa-B (TLR-NFκB) signaling in the liver of turbot fed a HLD and in turbot primary liver cells incubated with oleic acid (OA). Overexpression of *SOCS3* increased TG deposition via the increased production of mature sterol regulatory element binding protein 1 (*m*-SREBP-1). Knockdown of *SOCS3* in turbot primary liver cells resulted in normalized TG deposition and decreased *m*-SREBP-1 production. These results suggest that the HLD and OA can induce cytokine expression by activating the TLR-NFκB signaling pathways, resulting in increased SOCS3 expression. It is proposed that SOCS3 enhances *m*-SREBP-1 production, leading to TG deposition. These findings provide important new insights into the relationship between cytokine expression and TG deposition and mechanism of HLD-induced pro-inflammatory response, which could help to improve the health of farmed turbot and a better understanding of fish immunity.

1. Introduction

The use of high-lipid diets (HLD) is common in fish aquaculture because of its protein-sparing effect [1]. However, the use of these diets to feed farmed fish causes excessive lipid deposition [2,3] and the overexpression of pro-inflammatory cytokines [4], which negatively impacts fish normal immunity and affects their quality for human consumption.

Pro-inflammatory cytokines, such as tumor necrosis factor α (TNFα), stimulate the production of suppressors of cytokine signaling (SOCS), which modulate cytokine signaling through multiple mechanisms [5,6]. Studies in mammals have indicated that a relationship exists between pro-inflammatory cytokine overexpression and altered lipid metabolism through the activation of SOCS3. In mice, TNFα was reported to acutely and dramatically accelerate triglyceride (TG) accumulation. This increase was related to the increased production of the proteins, SOCS3 and sterol

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regulatory element binding protein 1c (SREBP-1c) [7]. In an obesity model, the production of SOCS3 and SREBP-1c were both increased while the inhibition of SOCS3 restored normal SREBP-1c levels, thus dramatically improving the symptoms of hypertriglyceridemia and eventually reducing TG deposition [8].

While TNFα is reported to be involved in lipid metabolism in fish adipose tissue (as reviewed in Ref. [9]), little else is known about the relationship between pro-inflammatory cytokines and altered lipid metabolism in fish. The sequence and immunological functions of the SOCS protein family are conserved across vertebrates (as reviewed in Ref. [10]). As in mammals, SOCS protein levels are increased by cytokine signaling in fish [11,12]. In addition, *SOCS3* gene expression levels are reported to be significantly increased in the liver of long-term fasting Arctic char, and it was concluded that leptin interacts with SOCS3 in this species to suppress lipolytic pathways [13]. In zebrafish, *SOCS3* has also been implicated in reduced lipid levels produced by a green tea extract [14]. Together, these studies implicate SOCS3 in lipid regulation and metabolism in fish – but how *SOCS3* functions in these processes remains poorly understood. Therefore, investigation of *SOCS3* activation mechanism and lipid metabolism could offer important insight into HLD-induced pro-inflammatory response and cytokines-caused lipid deposition.

Turbot (*Scophthalmus maximus*) is of high economic value, owing to its popularity for human consumption and its rapid growth, and it is increasingly being farmed by commercial aquaculture [15]. The use of a HLD in turbot has become common practice. Thus, turbot is a good model in which to investigate the mechanism of SOCS3 in fish lipid metabolism. This study was to determine how HLD induced SOCS3 expression and whether SOCS3 promotes TG deposition in turbot with the aim to uncover the mechanism of HLD-induced pro-inflammatory response and relationship between pro-inflammatory cytokines and lipid metabolism, which may help to improve understanding of fish immunity and lipid metabolism.

2. Materials and methods

2.1. Animals, diet formulation and animal husbandry

Animals, diet formulation and animal husbandry were as described previously [16]. Briefly, animal experiments were performed in a recirculating system at National Marine Science Research Center (Qingdao, China). Using fish meal, soybean meal, wheat gluten meal and casein as the main protein sources, and fish oil and soybean oil (1:1) as the main lipid source, two isonitrogenous (50% crude protein) but different lipid content diets were formulated: a control lipid diet (CON, lipid content, 11.87%) and a high-lipid diet (HLD, lipid content, 22.68%). The compositions of the two diets were analyzed (Table 1). Procedures for diet preparation and storage were described previously [17].

This study was performed in accordance with the Standard Operation Procedures (SOPs) of the Guide for the Use of Experimental Animals of Ocean University of China. All animal care and use procedures were approved by the Institutional Animal Care and Use Committee of Ocean University of China. Fish were anesthetized with MS222 (Sigma-Aldrich, USA) to minimize suffering before being assigned to cages and sampling.

After fasting for 24 h, fish (9.49 ± 0.03 g) were randomly divided into two tanks (300 L) with 35 fish per tank. Each diet was randomly divided into three portions and assigned to a tank. The fish were fed twice a day (08:00 and 18:00) to apparent satiation for 12 weeks.

2.2. Primary culture of turbot liver cells

Juvenile turbots were treated in sea water containing 1000 IU/mL penicillin and 1.2 mg/mL streptomycin (Sangon Biotech, China) for 24 h. Livers were removed and placed in sterile phosphate-buffered saline (PBS) containing penicillin and streptomycin. After washing with Leibovitz's L-15 medium, the liver tissue was minced into 1-mm^3 pieces and digested with 0.1% collagenase II solution (Sigma-Aldrich, USA) for 10 min. Cell pellets were re-suspended in complete medium consisting of Leibovitz's L-15 medium supplemented with 20% Gibco fetal bovine serum (Thermo Fisher Scientific, USA), 100 U penicillin and streptomycin, and 2 mM L-alanyl-L-glutamine (Thermo Fisher Scientific, USA). Cell suspensions were seeded in 6-well culture plates and incubated at 24 °C.

2.3. Oleic acid preparation

Oleic acid (Sigma, USA) was added to culture medium in the form of a BAS-FA complex, according to methods previously described [18]. Briefly, oleic acid (OA) was solubilized in ethanol at 10 mM containing 20 μM α-tocopheryl succinate (Sigma, USA) and stored at −20 °C. Before each assay, fatty acid-free BSA (Equitech-Bio, USA) was dissolved in L-15 medium containing 100 U/mL penicillin and 100 μg/mL streptomycin at pH = 7.2. After being dried under nitrogen, OA was added to the medium and sonicated for 5 min to result in a 150 μM OA solution. The solution was sterilized by filtration through 0.22-μm low-protein-binding filters (Pall, USA).

2.4. Cell collection

After discarding the culture medium, cells were washed twice with PBS, treated with 0.25% trypsin, and collected in centrifuge tubes. The cells were centrifuged at 500×g for 5 min, re-suspended with PBS, and re-centrifuged. Cell pellets were immediately frozen in liquid nitrogen and stored at −80 °C for further analysis.

2.5. RNA extraction and cDNA synthesis

At the end of the feeding trial, fish were fasted for 24 h, and five fish per tank were randomly collected and anaesthetized (MS222; Sigma, USA). The bloodless fish were sacrificed on ice packs to harvest livers, which were collected in 1.5-mL cryogenic microtubes (Sangon, China). Tissue was flash frozen in liquid nitrogen and stored at −80 °C prior to analysis.

Turbot livers were ground to powder in liquid nitrogen. To prevent melting, cell samples were put on ice. Next, Trizol reagent (Takara, Japan) was added into tubes containing tissue and cell samples, respectively, and total RNA was extracted according to the manufacturer's protocol. To remove residual genomic DNA, extracted RNA was treated with RNase-Free DNase (Takara, Japan) at 42 °C for 2 min. The integrity of the extracted RNA was determined by electrophoresis on a 1.2% agarose gel, and the concentration was measured using a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA). Total RNA with a 260/280 nm absorbance ratio from 1.8 to 2.0 was used for further experiments. The extracted RNA was reverse transcribed to first-strand cDNA using a PrimerScript™ RT reagent kit (Takara, Japan), according to the manufacturer's instructions.

2.6. In vitro transcription of mRNA encoding SOCS3 and eGFP

To produce capped mRNA by *in vitro* transcription, forward primer mSOCS3-F and reverse primer mSOCS3-R were designed to amplify *SOCS3* (Table 2). A PCR template for the cloning of SOCS3

was obtained as described in "RNA extraction and cDNA synthesis". In addition, forward primer meGFP-F and reverse primer meGFP-R were designed to amplify *eGFP* (which encodes enhanced green fluorescent protein) (Table 2). The PCR template for *eGFP* was plasmid PIRESE-eGFP (CloneTech, Japan). PCR products of expected size were excised from agarose gels and purified with a SanPrep Column DNA Gel Extraction Kit (Sangon, China). The purified DNA templates for *in vitro* transcription were further purified by phenol/chloroform extraction. *In vitro* synthesis of mRNA was conducted using a mMESSAGE mMACHINE® T7 ULTRA Transcription Kit (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. Purified mRNA was quantified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA) and diluted to 0.5 μg/μL in RNase-free H_2O. The prepared mRNA was stored at −25 °C until use.

2.7. Transfection of SOCS3

Liposome-mediated SOCS3 or eGFP transfection was performed when the confluence of primary turbot liver (TL) cells reached ~90% in the 6-well plates. The transfection reagent Lipofectamine® MessengerMax (Thermo Fisher Scientific, USA) was adopted to carry mRNA into the cells, and the procedures were performed according to the manufacturer's instructions. Briefly, 30 min before the transfection, the culture medium was discarded and replaced with 1.8 mL of fresh medium. Transfection reagent (5 μL) was diluted in 100 μL of Opti-MEM medium (Thermo Fisher Scientific, USA) to form a lipo-medium complex and was incubated at room temperature for 10 min. The complex was then mixed with mRNA (2.5 μg), which was pre-diluted in 100 μL of Opti-Mem medium. After incubation at room temperature for 5 min, the mRNA-lipid complex was added to the primary TL cells. The cells were incubated under optimum conditions after transfection.

Table 1
Formulation and analysis of the experimental diets (% dry weight).

Ingredients	Dietary lipid level (% dry weight)	
	11.87 (CON)	22.68 (HLD)
White fish meal [a]	27	27
Wheat gluten meal [a]	16	16
Casein [a]	15	15
Wheat meal [a]	8.3	8.3
Soybean meal [a]	18.8	18.8
Fish oil (FO)[b]	3.75	9
Soybean oil (SO)[b]	3.75	9
Soybean Phospholipid	2	2
Mineral premix [c]	0.5	0.5
Vitamin premix [d]	1	1
Choline chloride	0.13	0.13
Monocalcium phosphate	1	1
Calcium propionic acid	0.1	0.1
Ethoxyquin	0.05	0.05
Y_2O_3	0.04	0.04
Phagostimulant	1	1
α cellulose	10.5	0
Sodium alginate	1.5	1.5
Dietary analysis		
Crude protein	50.5	50.83
Crude lipid	11.87	22.68
Ash	8.24	7.9

[a] Composition of dry matter (%): White fish meal: crude protein 70.22, crude lipid 5.85; Wheat gluten meal: crude protein 83.95, crude lipid 1.28; Casein: crude protein 89.44, crude lipid 1.46; Wheat meal: crude protein 16.03, crude lipid 3.32; Soybean meal: crude protein 51.36, crude lipid 1.79.

[b] Fatty acid composition (% TFA): FO: SFA 23.94, MUFA 23.65, C18:2n-6 4.18, C18:3n-3 1.78, EPA 3.45, DHA 10.57; SO: SFA 16.55, MUFA 24.91, C18:2n-6 51.25, C18:3n-3 6.68. FO and RO obtained from Great seven Bio-Tech and Jin Long Yu.

[c] Mineral premix (mg/kg diet): NaF, 2; KI, 0.8; $CoCl_2 \cdot 6H_2O$ (1%), 50; $CuSO_4 \cdot 5H_2O$, 10; $FeSO_4 \cdot H_2O$, 80; $ZnSO_4 \cdot H_2O$, 50; $MnSO_4 \cdot H_2O$, 60; $MgSO_4 \cdot 7H_2O$, 1200; $Ca(H_2PO_3)_2 \cdot H_2O$, 3000; zeolite, 15.55 g/kg diet.

[d] Vitamin premix (mg/kg diet): thiamin, 25; riboflavin, 45; pyridoxine HCl, 20; vitamin B12, 0.1; vitamin K3, 10; inositol, 800; pantothenic acid, 60; niacin acid, 200; folic acid, 20; biotin, 1.20; retinol acetate, 32; cholecalciferol, 5; alpha-tocopherol, 120; ascorbic acid, 200.

2.8. In vitro synthesis of siRNA

Four pairs of oligonucleotides (Sn-SOCS3-F and Sn-SOCS3-R, As-SOCS3-F and As-SOCS3-R, Sn-eGFP-F and Sn-eGFP-R, As-eGFP-F and As-eGFP-R, sequences listed in Table 2) were synthesized for *in vitro* transcription and preparation of dsRNA. Single-strand RNA was synthesized using a MEGAscript T7 kit (Thermo Scientific, USA) according to the manufacturer's instructions. RNA was quantified and diluted in RNase-free water to 1 μg/μL. To form dsRNA, two pairs of complementary strands were hybridized by heating at 75 °C for 5 min and then cooled to room temperature.

For siRNA preparation, approximately 10 μg of dsRNA was digested with RNase III (New England Biolabs, USA) for 20 min at 37 °C in 1 × Short Cut reaction buffer and 20 mM $MnCl_2$, with a ratio of 2 U RNase III per μg dsRNA [19]. The prepared siRNA was quantified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA) and diluted to 1 μg/μL in RNase-free H_2O. The prepared mRNA was stored at −80 °C until use.

2.9. SOCS3 knockdown

SOCS3 knockdown was performed using Lipofectamine® RNAi-Max® (Thermo Scientific, USA) according to the protocol supplied by the manufacturer.

2.10. Triglyceride content determination

Triglycerides (TG) in the liver and primary TL cells were analyzed by colorimetric and enzymatic assays following the manufacturer's protocols (Serum Triglyceride Determination Kit, Sigma-Aldrich, USA).

2.11. TNFα recombinant protein biosynthesis

Tumor necrosis factor alpha from turbot (TNFα, GenBank: FJ654645.1) complete coding sequence was synthesized by PAS (PCR Accurate Synthesis) method. PCR product was inserted in to expression vector pCzn1 (Zhongding Biotechnology, China) at restriction enzyme cutting site of NdeI and XbaI. Recombinant plasmid (pCzn1-FJ654645.1) was transformed into clone strain TOP10 (Zhongding Biotechnology, China). TNFα protein in supernatant that induced by isopropyl-β-*d*-thiogalactoside (IPTG) was detected by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). TNFα protein was purified by nickel column. Endotoxin was removed for further purification. Half maximal inhibitory concentration (24 h) of purified TNFα protein was 280.61 μg/mL.

2.12. NFκB signaling inhibition

To investigate whether OA induced SOCS3 production was dependent on NFκB signaling activation or not, SN50 (Enzo life science, Switzerland) was supplemented in the medium to inhibit NFκB signaling activation. TL cells were pretreated with OA (150 μM) for 12 h. Then the medium was replaced by fresh medium that supplemented with SN50 or SN50M (the control, Enzo life science, Switzerland). Incubated with fresh medium for 15 min, cells were collected for western blotting.

Table 2
Sequences of PCR primers for gene cloning and RT-qPCR.

Primer names	Forward and Reverse primers sequence (5′ to 3′)	Tm (°C)	Fragment (bp)	PCR efficiency (%)	Correlation coefficient
m-SOCS3-F	GCTAATACGACTCACTATAGGGACAGGCCACCATGGTAACTC	65	650	–	–
m-SOCS3-R	TTAGATCGGCGCATCGTACTCCTGGAGGAACTCCTTCAGCGGATGAGG				
m-eGFP-F	GCTAATACGACTCACTATAGGGACAGGCCACCATGGTGAGCA	68	752	–	–
m-eGFP-R	TTACTTGTACAGCTCGTCCATGCCGAGAGTGATCCCGGCGGCGGTCACG				
Sn-*SOCS3*-F	GCTAATACGACTCACTATAGGGACAGGCCACCATGGTAACTC	65	650	–	–
Sn-*SOCS3*-R	TTAGATCGGCGCATCGTACTCCTGGAGGAACTCCTTCAGCGGATGAGG				
As-*SOCS3*-F	ATGGTAACTCACAGCAAGTTTGACTCCGCGATGAGCAGCAG	69	650	–	–
As-*SOCS3*-R	GCTAATACGACTCACTATAGGGACAGGCCACCTTAGATCG				
Sn-*eGFP*-F	GCTAATACGACTCACTATAGGGACAGGCCACCATGGTGAGCA	68	752	–	–
Sn-*eGFP*-R	TTACTTGTACAGCTCGTCCATGCCGAGAGTGATCCCGGCGGCGGTCACG				
As-*eGFP*-F	ATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATC	66	752	–	–
As-*eGFP*-R	GCTAATACGACTCACTATAGGGACAGGCCACCTTACTTGTA				
RT-*TLR22*-F	TGACTGTGATAACGCCTGGT	56	150	98	0.91
RT-*TLR22*-R	ACATCTACTGAGCAGGACCG				
RT-*MyD88*-F	CCCAATGGTAGCCCTGAGAT	56	133	101	0.97
RT-*MyD88*-R	CATCTCGGTCGAACACACAC				
RT-*IL-1β*-F	GCGACATGGTGCGATTTCTG	58	168	96	0.96
RT-*IL-1β*-R	GCTGGATGCTGAAGGTCTGG				
RT-*TNFα*-F	GGGGTGGATGTGGAAGGTGA	56	196	94	0.99
RT-*TNFα*-R	CTTGGCATTGCTGCTGATTT				
RT-*SOCS3*-F	ACTCGCAGTGGGAATATGTTCTAC	56	179	99	0.98
RT-*SOCS3*-R	AGGAACTCCTTCAGCGGATG				
RT-*β-actin*-F	GTAGGTGATGAAGCCCAGAGCA	56	204	98	0.992
RT-*β-actin*-R	CTGGGTCATCTTCTCCCTGT				

Primer names headed "m" are designed for the amplification of mRNA templates, and those marked "Sn" and "As" are designed for the preparation of double-stranded RNA. Underlined sequence: T7 promoter site; *eGFP*: enhanced Green Fluorescent Protein; Primers labeled "RT" are designed for RT-qPCR. *TLR22*: Toll Like Receptor 22; *MyD88*: myeloid differentiation factor 88; *IL-1β*: interleukin 1β; *TNFα*: tumor necrosis factor α; *SOCS3*: suppressor of cytokine signaling 3.

2.13. Real-time quantitative polymerase chain reaction (RT-qPCR)

Real-time quantitative polymerase chain reaction (RT-qPCR) was performed as previously described by Zuo et al. [20] in a quantitative thermal cycle (Eppendorf, Germany). Each sample was tested in triplicate. Primers of the reference gene, *β-actin*, were designed according to the published turbot sequences (Table 2). The comparative C_T method ($2^{-\triangle\triangle CT}$ method) was adopted to calculate the expression of target genes, and the obtained value indicated the n-fold difference relative to the standard [21].

2.14. Antibody preparation and western blotting

To prepare a polyclonal SOCS3 antibody, two adult New Zealand rabbits were immunized via injection with antigen polypeptide (sequence: DSAMSSSPVDSNMRL) mixed with complete Freund's adjuvant. The rabbits were boosted at 20, 34, and 48 days after the initial immunization. Rabbits were sacrificed 8 days after the last boost, and sera were obtained from the blood. The antibody validation was examined by western blotting according to previous description [22].

Nuclear and cytosolic fractions were extracted using the NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. Protein content was quantified using a Bradford Protein Assay Kit (Beyotime Institute of Technology, China). Western blot protocols were used as described in a previous study [4].

Polyclonal anti-Lamin B antibodies was obtained from Santa Cruz Biotechnology (USA), whereas anti-SREBP-1, anti-IKKα/β, anti phospho-IKKα/β, anti-IκBα, anti phospho-IκBα, and anti-p65 antibodies were purchased from Cell Signaling Technology (USA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and HRP-conjugated secondary antibodies were obtained from Golden Bridge Biotechnology (China).

2.15. Statistical analysis

Densitometry after Western blotting was quantified by Imagine J and then normalized by GAPDH or Lamin B. Statistical analysis was performed in SPSS 17.0 (SPSS Inc., USA). The data were subjected to a one-way analysis of variance (ANOVA) followed by Tukey's multiple-range test. For statistically significant differences, $P < 0.05$ was required. The results are presented as the mean ± S.E.M. (standard error of mean).

3. Results

3.1. Increased TG deposition is associated with increased SOCS3 and m-SREBP-1 expression in turbot liver and primary turbot liver cells

In this study, to investigate the relationship between TG deposition and SOCS3 production, a high-lipid diet (HLD) and oleic acid (OA) were adopted to induce TG accumulation in liver of turbot and primary turbot liver (TL) cells, respectively. Juvenile turbot were fed a HLD, and the TG content of their livers was assessed and found to have increased after 12 weeks on the HLD, relative to control diet (CON) with 11.87% lipid (dry weight, Table 1) ($P < 0.05$, Fig. 1a). In addition, primary turbot liver (TL) cells were incubated with OA (150 μM), and after 12 h of treatment were found to have increased TG content relative to untreated controls ($P < 0.001$), (Fig. 1a). Western blot data indicated that in the livers of HLD-fed turbot, SOCS3 and mature sterol regulatory element binding protein 1 (*m*-SREBP-1) protein levels were significantly increased relative to the control (CON) group ($P < 0.05$) (Fig. 1b). Similarly, when TL cells were incubated with OA, SOCS3 and *m*-SREBP-1 levels were significantly increased respectively, compared to BSA-treated controls ($P < 0.001$) (Fig. 1b). Taken together, these results suggest that the HLD and OA can result in increased SOCS3 and *m*-SREBP-1 production, and TG deposition.

3.2. SOCS3 overexpression and knockdown: effect on TG deposition and m-SREBP-1 levels

To investigate the relationship between SOCS3 and TG deposition, TL cells were transfected by mRNA encoding *SOCS3* and siRNA targeting *SOCS3*, respectively. TL cells transfected with mRNA

encoding SOCS3 (SOCS3+) had significantly increased SOCS3 protein levels 36 h after transfection, when compared with the control (CON1) group, which was transfected with mRNA encoding *eGFP* ($P < 0.01$) (Fig. 2a). Moreover, when TL cells were transfected with an siRNA targeting *SOCS3* (SOCS3-), SOCS3 protein expression was significantly reduced 36 h after transfection, compared with that of the CON2 group, which was transfected with an siRNA targeting *eGFP* ($P < 0.001$) (Fig. 2a).

TG content significantly increased in the SOCS3+ group when compared with CON1 ($P < 0.01$) and, conversely, significantly decreased in SOCS3- when compared with CON2 ($P < 0.001$) (Fig. 2b).

Western blot data indicated that *SOCS3* overexpression in the SOCS3+ group led to significant elevation of *m*-SREBP-1 protein levels ($P < 0.05$) (Fig. 2a). Conversely, *m*-SREBP-1 protein levels were significantly reduced following *SOCS3* knockdown, relative to levels in the *eGFP* siRNA control (CON2) ($P < 0.01$) (Fig. 2a).

These results indicated that overexpression of *SOCS3* may increased TG deposition via the increased production of *m*-SREBP-1. Knockdown of *SOCS3* in turbot primary liver cells resulted in decreased TG deposition and decreased *m*-SREBP-1 production.

3.3. Toll-like receptor-NFκB signaling increases in response to HLD and OA

To investigate the relationship between HLD and pro-inflammation response, toll-like receptor-NFκB (TLR-NFκB) signaling related proteins and genes were determined in the liver of turbot fed the HLD and in TL cells incubated with OA. Western blotting results indicated that *p*-IKKα/β, and n-p65 were all significantly increased, while *p*-IκBα was significantly decreased in the liver of turbot fed the HLD and in TL cells incubated with OA (Fig. 3a). No significant difference was observed among IKKα/β, IκBα and t-p65 protein levels in all treatment groups ($P > 0.05$). HLD and OA both significantly increased the expression levels of the genes encoding toll like receptor 22 (*TLR22*), myeloid differentiation factor 88 (*MyD88*), and the cytokines, *TNFα* and *IL-1β* ($P < 0.05$) (Fig. 3b and c). These results indicated HLD and OA inducted pro-inflammatory response by activating TLR-NFκB signaling in the liver of turbot fed the HLD and in TL cells incubated with OA.

3.4. SOCS3 induction by cytokines in TL cells

To investigate whether SOCS3 could be induced by cytokines or not, TNFα recombinant protein was added into the cell culture medium. After exposure to 0.5 μg/mL TNFα, the expression of *SOCS3* significantly increased in TL cells over time, peaking at 2 h after treatment (Fig. 4a). The production of SOCS3 reached the highest level at 1–4 h after 0.5 μg/mL TNFα treatment (Fig. 4b). Moreover, *SOCS3* expression levels were dependent on the dose of TNFα, as 0.1 μg/mL, 0.5 μg/mL, 1 μg/mL and 2 μg/mL doses all increased *SOCS3* expression within 2 h after administration, and the expression levels were proportional to the TNFα dose (Fig. 4c and d). These results suggested that SOCS3 production could be induced by cytokines, such as TNFα.

3.5. SOCS3 regulation by NFκB signaling

To investigate whether SOCS3 production was depend on the activation of NFκB signaling or not, TL cells were pretreated with OA (150 μM) for 12 h, then incubated with 25–100 μg/mL NFκB inhibitor SN50 for 15 min. Results indicated that SN50 (25–100 μg/mL) significantly reduced the production of nucleus p65 that induced by OA, rather than the control, SN50M in TL cells (Fig. 5a). SN50 (50–100 μg/mL) significantly reduced the production of

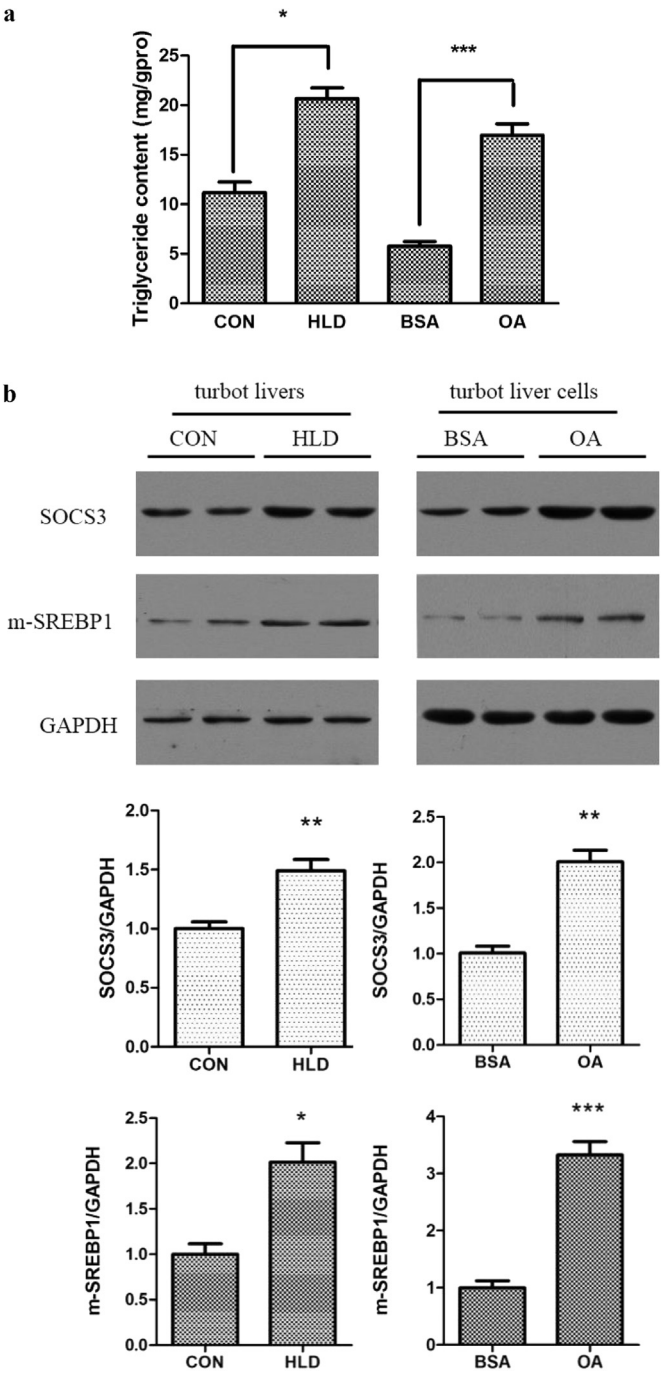

Fig. 1. High-lipid diet (HLD)-and oleic acid (OA) induce triglyceride (TG) deposition and suppressor of cytokine signaling 3 (SOCS3) and mature sterol regulatory element binding protein 1 (*m*-SREBP-1) proteins production. Turbot primary liver (TL) cells were cultured with L-15 medium (without serum) for 8 h. Afterwards, culture medium was replaced by assay medium supplemented with 150 μM OA. After incubated for 12 h, cells were collected for further study. (a) TG deposition in turbot liver (n = 3) and turbot primary liver (TL) cells (n = 6). (b) SOCS3 and *m*-SREBP-1 protein levels in the liver of turbot (n = 3) fed a high-lipid diet (HLD) and in TL cells (n = 6) treated with oleic acid (OA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal reference protein. CON: control diet; BSA: bovine serum albumin, the control. Data are expressed as A.U. of the western blots and are depicted as a ratio of SOCS3 to GAPDH, *m*-SREBP-1 to GAPDH. Data are presented as the mean ± S.E.M. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

SOCS3 protein levels in TL cells incubated with OA (Fig. 5b). These results indicated that the production of SOCS3 was depended on NFκB signaling activation. The inhibition of NFκB signaling results

in the reduction of SOCS3 production.

4. Discussion

HLD is widely used in aquaculture for commercial purposes, which results in excessive TG deposition and chronic inflammation [2,4]. Numerous studies have reported a correlation between TG deposition and cytokines production in mammals [23,24]; however, little is known about their relationships in fish. Given that SOCS proteins are conserved across vertebrates, we hypothesized that SOCS3 could provide a link between immunity and lipid metabolism in fish, as in mammals [10]. By investigating links between immunity and the regulation of TG deposition by SOCS3 in fish, we might be able to provide better understanding on the mechanism of HFD-induced fat deposition and abnormal immune functions.

Previous studies have indicated that a HLD fed to mammals and the exposure of mammalian cells to a monounsaturated fatty acid *in vitro* can induce TG deposition [25,26]. In this study, HLD and OA were employed to induce excessive TG deposition *in vivo* and *in vitro*, respectively. TG content increased in the livers of turbot fed a HLD and TL cells treated with OA, relative to controls, which indicate that HLD- and OA can induce excessive TG deposition. SREBPs are important transcription factors that regulate fatty acid and cholesterol metabolism in liver [27]. In sterol depletion, SREBPs are cleaved and become mature forms (*m*-SREBP) to bind sterol regulatory elements (SREs) [28,29] and/or E-box sequences and then activate the target gene expression. SREBP-1 protein, a positive regulator of TG deposition [30,31], was observed to increased in the liver of turbot fed HLD and TL cells incubated with OA, which may accounted for the increase in TG content. SOCS3 protein levels were also elevated in the livers of turbot fed the HLD and in TL cells incubated with OA, which is in agreement with results from mammalian obesity models [32,33]. These results show that HLD and OA cause an increase in SOCS3 levels and that this increase is associated with excessive TG deposition in turbot, as has been reported in mammals.

To investigate how SOCS3 regulates TG deposition in turbot, mRNA encoding *SOCS3* was transfected into TL cells. This approach overcomes the obstacle of nuclear membranes and thus improves the transfection efficiency in primary cells [34,35]. The overexpression of SOCS3 in TL cells incubated with OA increased both TG deposition and *m*-SREBP-1 protein expression relative to CON1, which was transfected with mRNA encoding *eGFP*. This was in accordance with the findings of Hang [36] and Ueki [8], who reported that SOCS3 induced SREBP-1c-related TG deposition in mouse. Conversely, TG deposition was decreased by *SOCS3* knockdown in TL cells incubated with OA, with a concomitant decrease in *m*-SREBP-1 protein levels, relative to CON2, which was transfected with siRNA targeting *eGFP*. Similar results have been reported in mammals, in which antisense targeting of *SOCS3* reduced TG deposition [32,37]. However, it is worth noting that in some mammalian studies, *SOCS3* depletion resulted in insulin and leptin resistance, which promoted the deposition of TG [38,39]. Similarly, in a recent zebrafish study, hepatic steatosis and insulin resistance were observed after the depletion of *SOCS1a*, which is believed to be functionally similar to *SOCS3* [40]. These conflicting results might be attributable to the differences between a SOCS gene being knocked down or knocked out. The deletion (knockout) of *SOCS3* or *SOCS1a* in the liver eliminates the response and feedback to cytokines, thus promoting chronic inflammation, TG deposition, and insulin and leptin resistance through multiple pathways [38,40]. It is suspected that the mechanism behind the correlation between *SOCS3* overexpression and increased TG deposition is related to

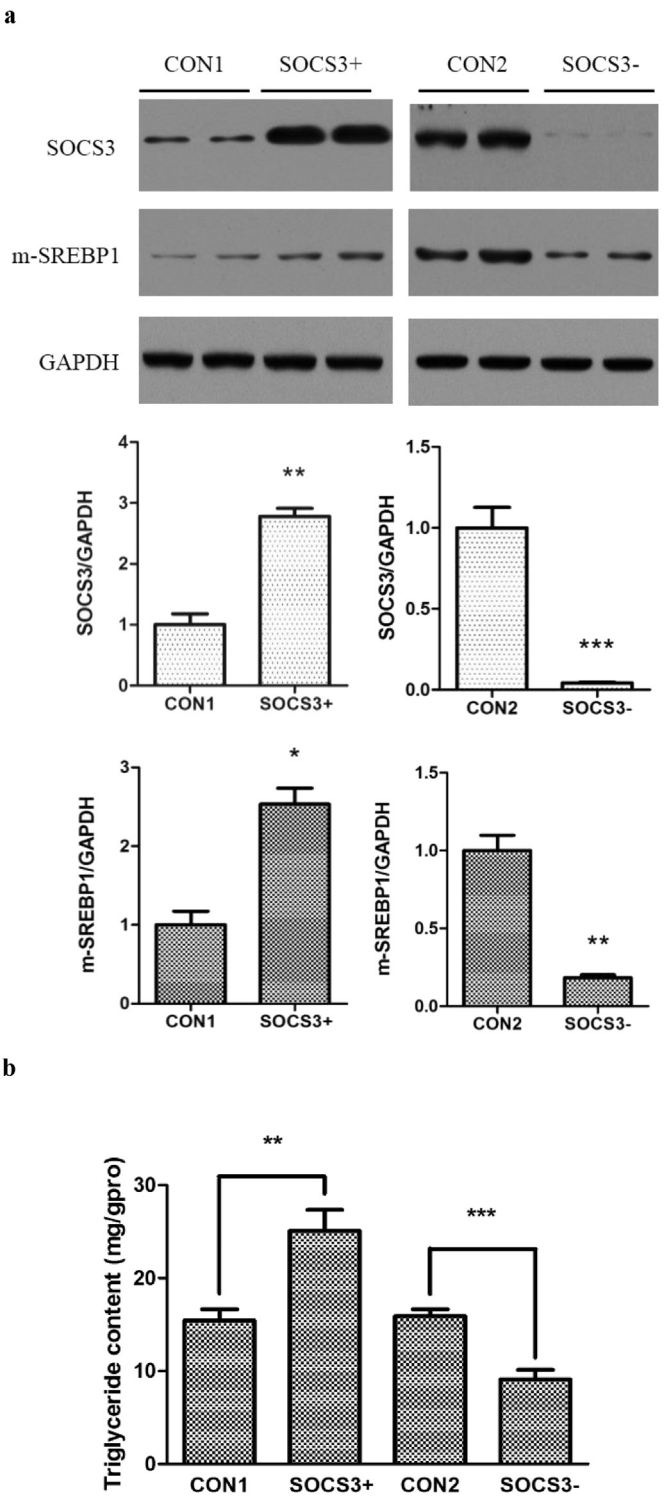

Fig. 2. Effects of *SOCS3* on the regulation of TG deposition and related protein expression in TL cells. (a) Western blot analysis of SOCS3 and TG deposition-related protein *m*-SREBP-1production in primary turbot liver (TL) cells following incubation with 150 μM OA for 12 h after transfection with: (left panel) mRNA encoding *eGFP* (CON1) or *SOCS3* (SOCS3+); or (right panel) with siRNA targeting *eGFP* (CON2) or *SOCS3* (SOCS3-). Protein levels were assayed following *SOCS3* overexpression (SOCS3+) or knockdown (SOCS3-) for SOCS3 and *m*-SREBP-1. Data are expressed as A.U. of the western blots and are depicted as a ratio of SOCS3 to GAPDH, *m*-SREBP-1 to GAPDH (n = 6). (b) Quantification of TG levels in TL cells treated with mRNA encoding *SOCS3* (SOCS+) or with *SOCS3* siRNA (SOCS-). Data are presented as the mean ± S.E.M (n = 6). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Abbreviations: SOCS3, suppressor of cytokine signaling 3; *m*-SREBP-1, mature sterol regulatory element binding protein 1.

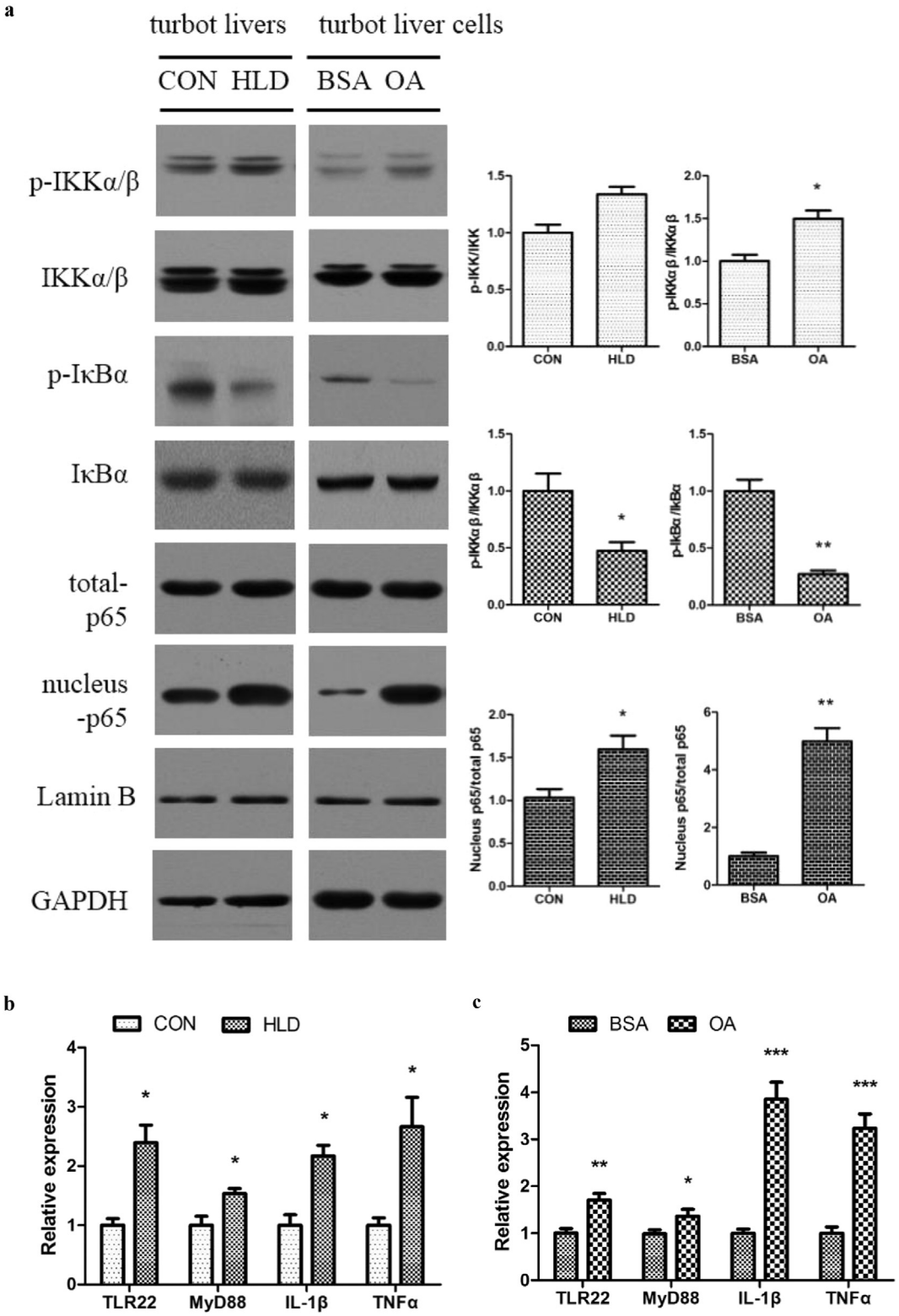

Fig. 3. High-lipid diet and oleic acid lead to increased TLR-NFκB signaling. (a) Western blot analysis of proteins involved in NF-κB signaling in the liver of turbot fed a CON diet or HLD (left gel) (n = 3). Western blot analysis of proteins involved in NF-κB signaling in TL cells incubated with BSA or OA (right gel) (n = 6). Data are expressed as A.U. of the western blots and are depicted as a ratio of *p*-IKKα/β to IKKα/β, *p*-IκBα to IκBα and nucleus p65 to total p65. (b) Quantification of a RT-qPCR analysis of *TLR22*, *MyD88*, *IL-1β* and *TNFα* expression levels in the livers of turbot fed a control (CON) diet or a high-lipid diet (HLD) (n = 3). (c) Quantification of a RT-qPCR analysis of *TLR22*, *MyD88*, *IL-1β* and *TNFα* expression in primary turbot liver (TL) cells incubated with BSA (control) or oleic acid (OA) (n = 6). The data are presented as the mean ± S.E.M. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

p-STAT3, which is suppressed by SOCS3 [41,42]. Mammalian study found that SOCS3 expression elevated TG deposition through a decrease in p-STAT3 protein, which enhanced SREBP-1 promoter activity [43].

To explain the increased levels of SOCS3 in the liver of turbot fed the HLD and in TL cells treated with OA, TLR-NFκB signaling was

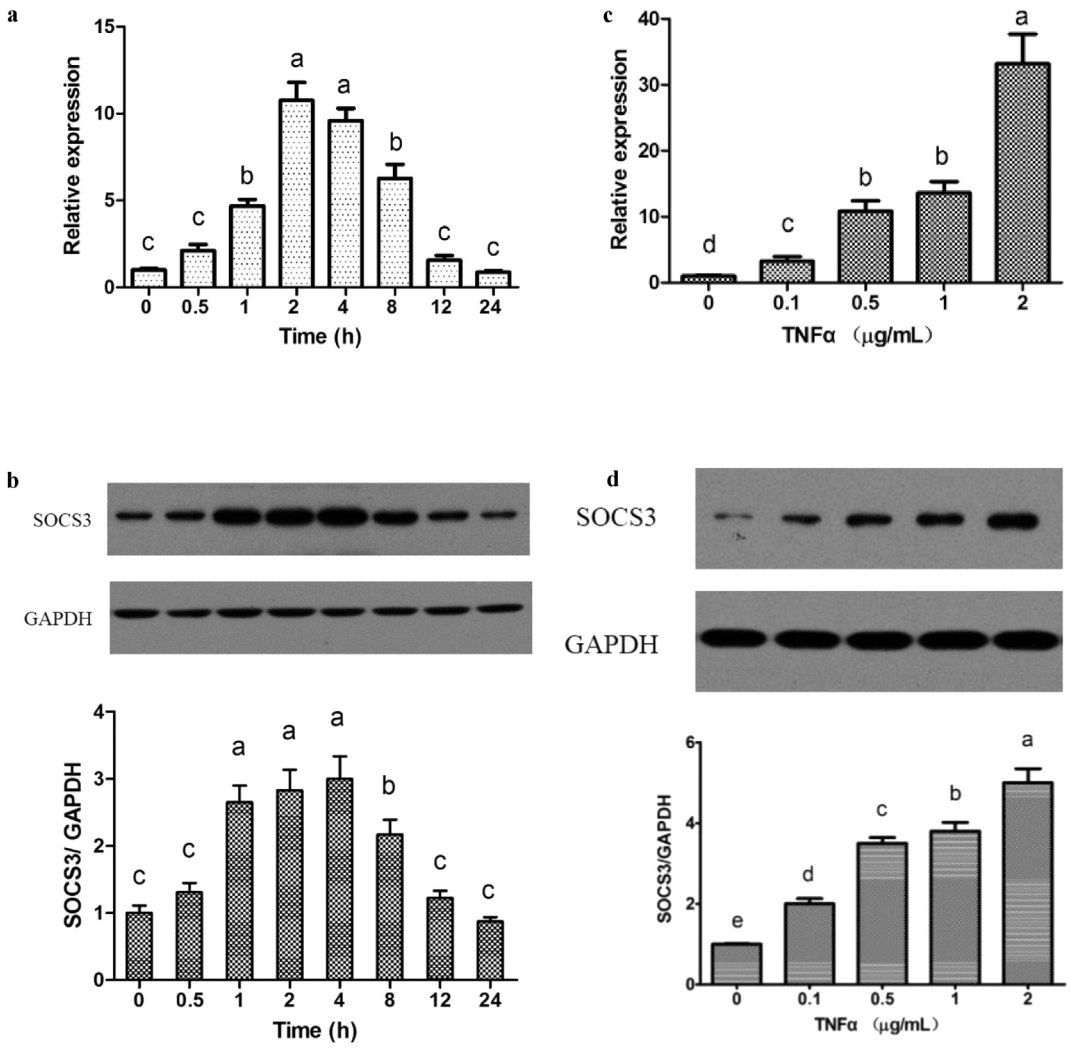

Fig. 4. SOCS3 response to TNFα in primary turbot liver cells. (a) Relative *SOCS3* expression in response to 0.5 μg/mL TNFα in primary turbot liver (TL) cells. (b) SOCS3 production in response to 0.5 μg/mL TNFα in primary turbot liver (TL) cells. Data are expressed as A.U. of the western blots and are depicted as a ratio of SOCS3 to GAPDH. (c) Relative *SOCS3* expression in response to 0–2 μg/mL TNFα in TL cells. (d) SOCS3 production in response to different dose of TNFα in TL cells. Data are expressed as A.U. of the western blots and are depicted as a ratio of SOCS3 to GAPDH. Letters above each bar share same letter means no significant difference among treatments at the $P > 0.05$ level (n = 6).

studied because this classic immunity pathway is associated with cytokine production and might induce the overexpression of *SOCS3* [11]. In the liver of turbot fed the HLD and in TL cells incubated with OA, *TLR22* and the TLRs downstream adaptor molecule, *MyD88*, were both significantly increased. This accords with a previously published mammalian study, which reported that HLD and monounsaturated fatty acid induced the overexpression of TLR4, due in part to the production of excessive reactive oxygen species (ROS) [44]. Our group has also previously reported that *TLR22* levels in the yellow croaker fish (*Larimichthys polyactis*) correlated positively with ROS production following low or moderate n-3 HUFA treatment [45]. TLRs transmit extracellular signals to activate NFκB signaling, which was manifested as increase in *p*-IKKα/β, and n-p65 as well as decrease in *p*-IκBα in the liver of turbot fed HLD and TL cells incubated with OA [46]. Previous studies in mammals have reported that NFκB signaling can stimulate the expression of the pro-inflammatory cytokines [47]. In this study, activated NFkB signaling appears to stimulate the expression of *TNFα* and *IL-1β*. Afterwards, TNFα stimulated the production of SOSC3 in mRNA and protein levels, which was in accordant with finding in turbot macrophages derived from head kidney [45]. Moreover, the inhibition of NFκB signaling decreased the production of SOCS3 in TL cells incubated with OA. These results indicated that SOCS3 production was dependent on the activation of NFκB signaling. It is suggested that the activation of NFκB signaling increased the expression of TNFα and then activated the production of SOCS3. These results indicated that HLD and OA might induced pro-inflammatory response by activating TLRs-NFkB signaling which appears to stimulate the overexpression of TNFα and then increased SOCS3 protein production.

Taken together, we propose possible mechanisms for HLD-induced pro-inflammatory response and how pro-inflammatory cytokines might cause TG deposition via the activation of SOCS3 in turbot. In this model, HLD and OA activate the TLR-NFκB signaling pathway, resulting in TNFα production. TNFα promote the increases expression of SOCS3, then lead to enhanced *m*-SREBP-1 production, resulting in the up-regulation of TG deposition. This findings improve understanding of HLD-caused pro-inflammatory response by investigating SOCS3 on the one hand. On the other hand, this finding provide important insight into the cytokines aggravate lipid deposition in fish fed HLD.

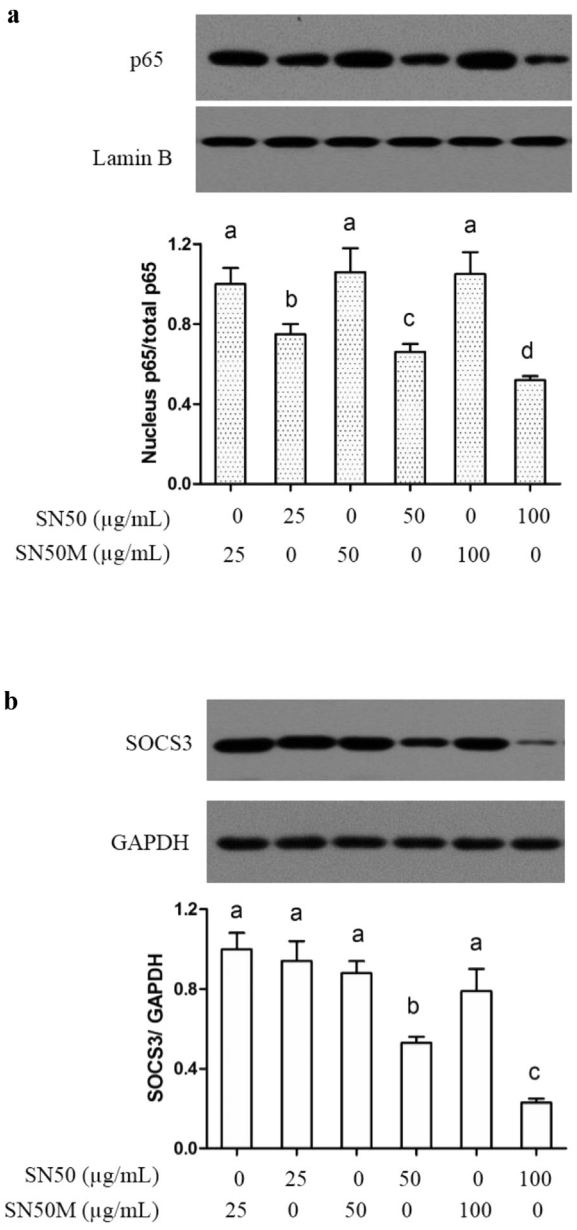

Fig. 5. NFκB signaling activation relate to OA induced SOCS3 production. (a) Nucleus p65 protein inhibited by SN50. TL cells were pretreated with OA (150 μM) for 12 h. The cells were then incubated with 25–100 μg/mL NFκB inhibitor SN50 and SN50M (the control) for 15 min. Data are expressed as A.U. of the western blots and are depicted as a ratio of nucleus p65 to Lamin B. (b) SOCS3 production decreased by NFκB signaling inhibitor. Data are expressed as A.U. of the western blots and are depicted as a ratio of SOCS3 to GAPDH. Letters above each bar share same letter means no significant difference among treatments at the $P > 0.05$ level ($n = 6$).

Author contributions statement

Kangsen Mai, Qinghui Ai, Huarong Guo and Rui Nian designed the study. Peng Tan, Mo Peng and Dongwu Liu performed the study. Peng Tan and Benoit Macq analyzed the data and wrote the paper. All authors read and approved the final manuscript.

Competing financial interests

The authors declare no competing financial interests.

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