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Adipose tissue contributes to hepatic pro-inflammatory response when dietary fish oil is replaced by vegetable oil in large yellow croaker (*Larimichthys crocea*): An *ex vivo* study

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ABSTRACT

The shortage of fish oil (FO) leads to the extensive use of vegetable oil (VO) in marine fish diets. High replacement percentage of dietary FO by VO induced pro-inflammatory response of adipose tissue (AT) and liver tissue (LT) in large yellow croaker (*Larimichthys crocea*). Mammalian studies showed that the secretion of cytokines by AT affected the immune response of LT. To investigate whether or not the inflammation response of LT is related to AT in large yellow croaker, LT and AT cells from fish fed FO diet (FOL and FOA) and VO diet (VOL and VOA) were co-cultured in a *trans*-well system, which resulted in an assembly of the two cells types sharing the culture medium but being separated by the membrane of the insert.

Co-culture of FOL and FOA was selected as the control group (FOL-FOA). Results indicated that, when compared with the control group, the expression of pro-inflammatory genes (*toll like receptors* [*TLRs*], *tumour necrosis factor α* [*TNFα*], *interleukin 1β* [*IL1β*], *suppressor of cytokine signalling 3* [*SOCS3*] and *cyclooxygenase 2* [*COX2*]) in FOL was significantly increased in the co-culture group of FOL and VOA (FOL-VOA), while the expression of anti-inflammatory genes (*arginase I* [*ArgI*] and *transforming growth factor β1* [*TGFβ1*]) in FOL was significantly depressed. On the contrary, a significantly depressed expression of pro-inflammatory genes (*TLRs*, *TNFα*, *IL1β* and *COX2*) and increased expression of anti-inflammatory genes (*interleukin 10* [*IL10*]) in VOL was observed in the co-culture group of VOL and FOA (VOL-FOA) when compared with the co-culture group of VOL and VOA (VOL-VOA). The change of immune-related gene expressions in LT cells was attributed to nuclear factor κB (NF-κB) signalling since the expression of the p65 protein was observed to show a similar trend to the expression of pro-inflammatory genes. It is speculated that dietary VO increased the secretion of cytokines, which induced pro-inflammatory response in LT cells. These *ex vivo* results indicate that AT plays a vital role in LT pro-inflammatory response in fish fed VO diet.

1. Introduction

The limited production of fish oil (FO) urges us to find some alternatives for aqua-feed industry. Vegetable oil (VO) is the most promising alternative because of its low price, high output, rich unsaturated fatty acids and low organic contaminant. However, high percentage of dietary FO replaced by VO has led to pro-inflammatory response in the liver tissue (LT) [1,2], adipose tissue (AT) [3], kidney [4–6] and

Abbreviations: FO, fish oil; VO, vegetable oil; AT, adipose tissue; LT, liver tissue; FOL, liver tissue cells from fish fed FO diet; FOA, adipose tissue cells from fish fed FO diet; VOL, liver tissue cells from fish fed VO diet; VOA, adipose tissue cells from fish fed VO diet; *TLR*, t*oll like receptor*; *MyD88*, *myeloid differentiation factor 88*; *TNFα*, t*umour necrosis factor α*; *IL1*β, int*erleukin 1β*; *SOCS3*, *suppressor of cytokine signalling 3*; *COX2*, cy*clooxygenase 2*; *IL10*, *interleukin 10*; *Arg*I, a*rginase I*; *TGFβ1*, t*ransforming growth factor β1*; NF-κB, nuclear factor κB; PBS, phosphate-buffered saline; DMEM/F12, Dulbecco's modified eagle medium/ Ham's F12 medium; FBS, fetal bovine serum; S.E.M, stand error of the means

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intestine [7] in some fish species. The health status of liver has attracted much attention when fish were fed diets with high VO inclusion, since liver is closely related to nutrient metabolism. Persistent studies focusing on antioxidant capacity [8,9] and toll like receptor-nuclear factor κB (TLRs-NF-κB) pathway [2] have been conducted to elucidate the mechanism of pro-inflammatory response induced by dietary VO in liver, which however has not been fully elucidated so far.

When investigating the mechanism of liver pro-inflammatory response in fish fed high VO diet, previous studies mainly focused on liver itself, but overlooked the organ-to-organ and tissue-to-tissue cross talks. Evidences from mammalian studies have proved that AT is not only a fat storage tissue, but also an endocrine tissue that influences other organs by secreting adipo-cytokines (reviewed by Masoodi M. [10]). 3T3-L1 cell and primary liver cell were co-cultured in an *ex vivo* study to simulate the cross talk between AT and LT in obesity model. Results indicated that pro-inflammatory gene expressions and insulin resistant levels both increased in LT cell, due to the secretion of cytokines such as TNFα and IL6 by 3T3-L1 cell [11]. Moreover, a recent study pointed out that the pro-inflammatory response in AT was one of the main causes of non-alcoholic fatty liver disease, insulin resistance and abnormal fat deposition [12]. In fish species, studies of AT mainly focus on the proliferation, differentiation [13,14] and metabolism [15,16], only a few study focus on the immune response of AT [17]. As previous studies found that dietary VO could induce the pro-inflammatory response of AT and LT in large yellow croaker [3], whether the pro-inflammatory response of LT is related to AT as in mammalian studies would be worthy of investigation. To our knowledge, no study has been conducted to investigate the mechanism of liver pro-inflammatory response induced by VO from the perspective of crosstalk between AT and LT.

In the present study, LT and AT cells from fish fed FO and VO diets were co-cultured in *trans*-well, which resulted in an assembly of the two cell types sharing the culture medium but being separated by the membrane of the insert. Afterwards, pro-inflammatory genes and NF-κB pathway marker protein in LT cells were both analyzed. The aim of the present study was to elucidate the role of AT in VO induced LT pro-inflammatory response in large yellow croaker.

2. Materials and methods

2.1. Animals, diet formulation and animal husbandry

Animals, diet formulation and animal husbandry were as described previously [18]. Briefly, animal experiments were performed in a net cage system at Xihu Harbor (Ningbo, China). Using soybean meal and defatted fish meal as the main protein sources, two iso-nitrogenous (41% crude protein) and iso-lipid (12% crude lipid) diets were formulated with the replacement of fish oil by vegetable oil being as follows: 0% replacement (FO) and 100% replacement (VO, soybean oil: linseed oil = 1:1). The approximate compositions were analyzed (Table 1). The content of different fatty acids in the experimental diets (mg/g) were determined (Table 2).

Disease-free and equal sizes of large yellow croaker were from a commercial farm in Ningbo, China. Before the experiment, fish was acclimatized by feeding the control diet for two weeks. After fasted for 24 h, fish (8.93 g ± 0.21 g) were randomly divided into 9 floating cages with 60 fish per cage. Each diet was randomly divided into 3 parts, and each part was randomly assigned to a net cage. Fish were fed twice a day to apparent satiation for 70 days. Husbandry was under appropriate conditions.

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 used procedures were approved by the Institutional Animal Care and Use Committee of Ocean University of China. Fish were anaesthetized with MS222 (Sigma-Aldrich, USA) to minimize suffering before being assigned to cages and sampling.

Table 1
Formulation of the experimental diets (% dry matter) [18].

Ingredients	FO[a]	VO[b]
Defatted white fish meal[c]	15	15
Soybean meal	32	32
Casein[d]	11	11
Wheat meal	26	26
Mineral premix[e]	2	2
Vitamin premix[f]	2	2
Attractant[g]	0.3	0.3
Mould inhibitor[h]	0.1	0.1
Lecithin	2.6	2.6
Fish oil	9	0
Soybean oil	0	4.5
Linseed oil	0	4.5
Total	100	100
dry %		
Crude protein	41.67	41.71
Crude lipid	12.85	12.76

[a] FO: Fish oil group.
[b] VO: blend of vegetable oil replacing fish oil at 100%.
[c] Defatted fish meal: 72.1% crude protein and 1.4% crude lipid; white fish meal was defatted with ethanol (fish meal: ethanol = 1:2 (w: v)) at 37°Cfor three replications.
[d] Casein: 88% crude protein and 1.3% crude lipid, Alfa Aesar, Avocado Research Chemicals Ltd, UK.
[e] Mineral premix (mg or g kg^{-1} diet): $CuSO_4 \cdot 5H_2O$ 10 mg; Na_2SeO_3 (1%) 25 mg; $ZnSO_4 \cdot H_2O$, 50 mg; $CoCl_2 \cdot 6H_2O$ (1%) 50 mg; $MnSO_4 \cdot H_2O$ 60 mg; $FeSO_4 \cdot H_2O$ 80 mg $Ca(IO_3)_2$ 180 mg; $MgSO_4 \cdot 7H_2O$ 1200 mg; zeolite 18.35 g.
[f] Vitamin premix (mg or g kg^{-1}diet): vitamin D 5 mg; vitamin K 10 mg; vitamin B12 10 mg; vitamin B6 20 mg; folic acid 20 mg; vitamin B1 25 mg; vitamin A 32 mg; vitamin B2 45 mg; pantothenic acid 60 mg; biotin 60 mg; niacin acid 200 mg; α-tocopherol 240 mg; inositol 800 mg; ascorbic acid 2000 mg; microcrystalline cellulose 16.47 g.
[g] Phagostimulant: Glycine/Betaine = 1:3.
[h] Preservative: Fumarate/Calcium pnpionabe = 1:1.

Table 2
The content of different fatty acids in the experimental diets (mg/g)[a] [18].

Fatty acid	FO	VO
C 14: 0	0.76	0.10
C 16: 0	4.51	3.13
C 18: 0	1.63	1.71
ΣSFA[b]	6.90	4.94
C 16: 1	1.08	0.06
C 18: 1	3.59	5.47
ΣMUFA[c]	4.67	5.53
C 18: 2n-6	4.35	12.66
C 20: 4n-6	0.12	0.04
Σn-6 PUFA[d]	4.47	12.70
C 18: 3n-3	0.43	6.98
C 20: 5n-3	1.25	0.06
C 22: 6n-3	1.85	0.08
Σn-3 PUFA[e]	3.53	7.12
Σn-3/Σn-6 PUFA	0.79	0.56
Σn-3 LC-PUFA	3.10	0.14
Total fatty acids	21.18	31.09

[a] Some fatty acids, of which the contents are minor, trace amount or not detected, such as C22: 0, C24: 0, C14: 1, C20: 2n-6, C20:3n-6, were not listed in the table.
[b] SFA: saturated fatty acid.
[c] MUFA: monounsaturated fatty acid.
[d] n-6 PUFA: n-6 poly-unsaturated fatty acid.
[e] n-3 PUFA: n-3 poly-unsaturated fatty acid.

2.2. Isolation and culture of primary lager yellow croaker liver tissue (LT) cells

At the end of the feeding trial, fish were fasted for 24 h and were randomly collected and anaesthetized with MS222 (Sigma, USA). Livers

of juvenile lager yellow croaker were sampled and placed in sterile phosphate-buffered saline (PBS) containing penicillin and streptomycin. After being washed with Dulbecco's modified eagle medium/Ham's F12 medium (1:1) (DMEM/F12) medium for twice, the liver tissue was minced into 1-mm^3 pieces and digested with 0.25% trypsin (Thermo Fisher Scientific, USA) for 10 min. After being neutralize by DMEM/F12 medium containing Gibco fetal bovine serum (FBS, Thermo Fisher Scientific, USA), cell pellets were re-suspended in complete medium consisting of DMEM/F12 medium supplemented with 20% Gibco FBS, 100 U penicillin and 100 μg/mL streptomycin, and 2 mM L-alanyl-L-glutamine (Thermo Fisher Scientific, USA). Cell suspensions were seeded in 6-well culture plates and incubated at 28 °C.

2.3. Isolation and culture of primary lager yellow croaker adipose tissue (AT) cells

Isolation and culture of primary large yellow croaker adipose tissue cells were as described previously with some changes [14]. Briefly, the adipose tissue was excised from the wall of abdomen. After being washed with PBS and minced into pieces, the tissue was digested with 0.2% collagenase II (dissolved in HBSS) at room temperature for 1 h with intermittent shaking. The cell suspension was then centrifuged at 1000 rpm for 10 min. The supernatant was discarded and the cell suspension was washed with medium for twice. Finally, cell suspension was re-suspended in a complete medium consisting of DMEM/F12 medium supplemented with 20% Gibco fetal bovine serum, 100 U penicillin and 100 μg/mL streptomycin, and 2 mM L-alanyl-L-glutamine. Cells were seeded in 6-well culture plates and incubated at 28 °C.

2.4. Co-culture of lager yellow croaker adipose tissue cells and liver tissue cells

Nunc™ polycarbonate membrane inserts in multi-dishes (1.5 mL, pore size 0.4 μm, Thermo Fisher Scientific, USA) were used to conduct co-culture experiments. Adipose tissue cells and liver tissue cells, collected from fish fed the experiment diet, were co-cultured to simulate the cross talks between adipose tissue and liver tissue *in vivo*. AT cells were cultured in *trans*-well chamber while LT cells in *trans*-well insert. Procedures were used according to previous study with some modifications [19]. Briefly, LT cells and AT cells were digested with 0.25% trypsin and counted with scepter handheld cell counter (Millipore, USA). LT cells (5×10^5) and AT cells (2×10^6) were seeded in 6-well membrane inserts and chamber, respectively. The final medium volume was adjusted to 1.5 mL. This resulted in an assembly of the two cell types sharing the culture medium but being separated by the membrane of the insert. Distance from the bottom of the culture dish to the membrane was 1.0 mm. Cells were maintained in DMEM/F12 medium containing 20% FBS. Liver tissue cells from fish fed FO diet (FOL) and adipose tissue cells from fish fed FO diet (FOA) were co-cultured to simulate the *in vivo* status of fish fed the FO diet, for short FOL-FOA. Liver tissue cells from fish fed VO diet (VOL) and adipose tissue cells from fish fed VO diet (VOA) were co-cultured to simulate the *in vivo* status of fish fed the VO diet, for short VOL-VOA. FOL and VOA, VOL and FOA were also co-cultured, respectively, to investigate the role of adipose tissue in modulating immune-related gene and protein expression. Co-culture lasted for 24 h in biochemical incubator at 28 °C. Co-culture experiment used three fish each diet and repeated for six times.

2.5. Cell collection

After the co-culture, the culture medium and *trans*-well chamber were both discarded, with LT cells in *trans*-well insert left. LT cells were washed twice with PBS, treated with 0.25% trypsin, and collected into centrifuge tubes. The cells were then centrifuged at $500 \times g$ 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.

2.6. RNA extraction and cDNA synthesis

Trizol reagent (Takara, Japan) of 1 mL was added for a sample, 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 reversely transcribed to first-strand cDNA using a PrimerScript™ RT reagent kit (Takara, Japan), according to the manufacturer's instructions.

2.7. 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 sequences in Table 3. The comparative CT method (2^-

Table 3
Primers for RT-qPCR.

Primer names	Forward and reverse primers sequence (5′ to 3′)	Tm (°C)	Fragment (bp)	PCR efficiency (%)	Accession No.
L-TLR1-F/R	TGTGCCACCGTTTGGATA/TTCAGGGCGAACTTGTCG	57	95	99	KF318376.1
L-TLR2-F/R	TCTGCTGGTGTCAGAGGTCA/GGTGAATCCGCCATAGGA	57	98	98	KJ820743
L-TLR3-F/R	ACTTAGCCCGTTTGTGGAAG/CCAGGCTTAGTTCACGGAGG	58	159	102	HQ589262
L-TLR7-F/R	ATGCAATGAGCCAAAGTCT/CATGTGAGTCAATCCCTCC	54	185	97	KC543351.1
L-TLR9-F/R	AACGGAGGTCACAGGAGG/TAGCACCACTGGACAGCAC	55	133	98	EU655705
L-TLR13-F/R	CCTCCTGTTTATGGTAGTGTCC/GCTCGTCATGGGTGTTGTAG	56	161	98	NM001303396
L-TLR22-F/R	TATGCGAGCAGGAAGACC/CAGAAACACCAGGATCAGC	54	132	96	GU324977
L-MyD88-F/R	TACGAAGCGACCAATAACCC/ATCAATCAAAGGCCGAAGAT	57	144	98	EU978950
L-IL1β-F/R	CATCTGGAGGCGGTGGAGGA/GGGACAGACCTGAGGGTGGT	57	119	100	KJ459927
L-TNFα-F/R	CGTCGTTCAGAGTCTCCTGC/TGTACCACCCGTGTCCCACT	58	189	99	EF165623
L-SOCS3-F/R	CAAGCTCCAGGAAAGTGGCT/CACATTGGATGCGCAGGTTC	60	176	96	XM010738599
L-COX2-F/R	TAAACCTCGAAGCGACACGA/GACTTTGTCAGAAGTTCTTTTTGTC	58	83	95	KP259877
L-IL10-F/R	AGTCGGTTACTTTCTGTGGTG/TGTATGACGCAATATGGTCTG	55	144	99	XM010738826
L-Arg I-F/R	AACCACCCGCAGGATTACG/AAACTCACTGGCATCACCTCA	58	119	99	XM019269015
L-TGFβ1-F/R	GCAACCACCGTACATCCTGA/ACCCCATGCAGTAATTGGCA	59	212	103	XM019273006
L-βactin-F/R	GACCTGACAGACTACCTCATG/AGTTGAAGGTGGTCTCGTGGA	58	136	100	GU584189

TLR, toll-like receptor; MyD88, myeloid differentiation factor 88; IL1β, interleukin 1β; TNFα, tumour necrosis factor α; SOCS3, suppressor of cytokine signalling 3; COX2, cyclooxygenase 2; ArgI, arginase I; TGFβ1, transforming growth factor β1.

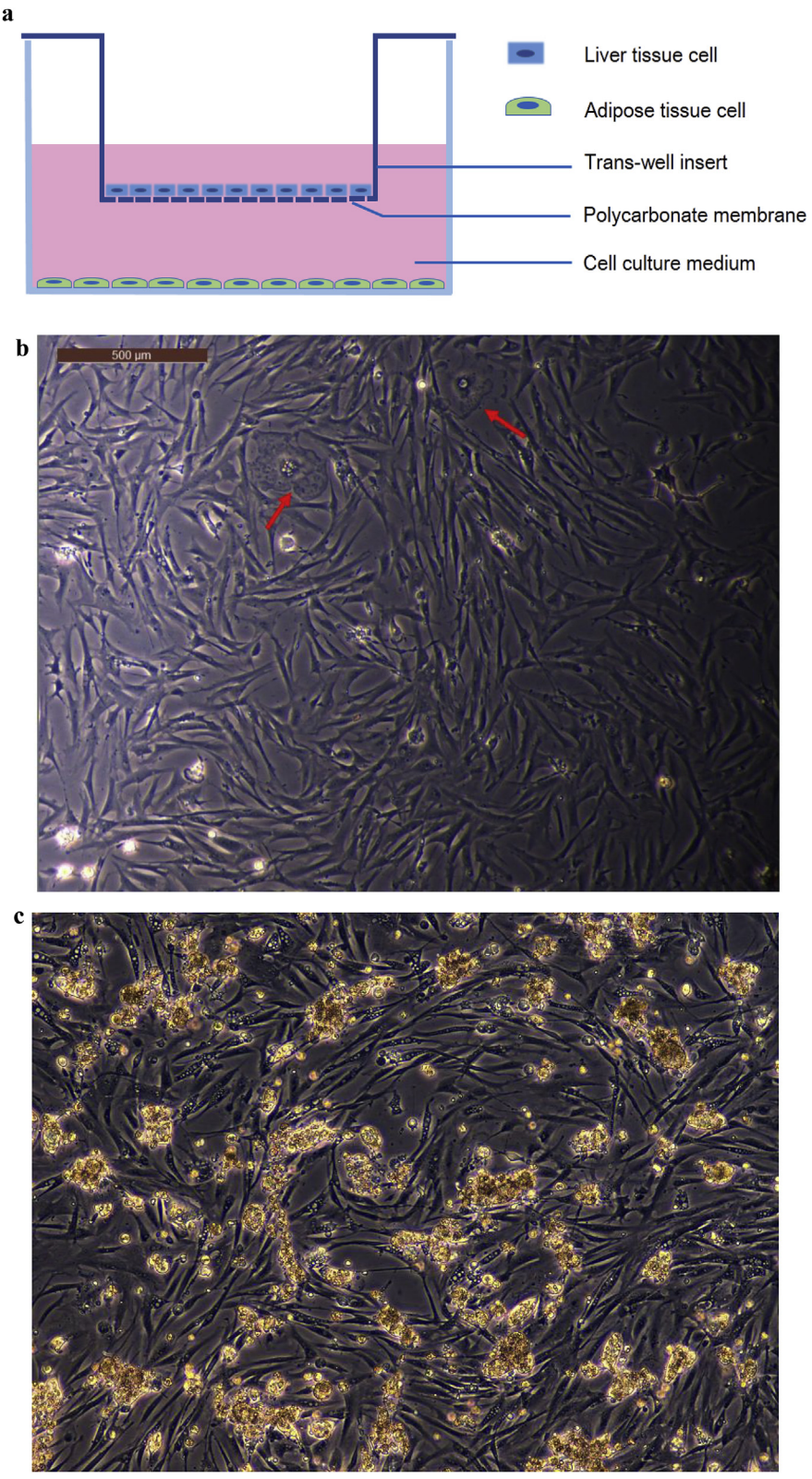

Fig. 1. Diagram of LT cells and AT cells co-culture system. Fig. 1a show the co-culture system in *trans*-well. LT cells (5×10^5) and AT cells (2×10^6) were seeded in 6-well membrane inserts and chamber, respectively. The final medium volume was adjusted to 1.5 mL. *Trans*-well resulted in an assembly of the two cells types sharing the culture medium but being separated by the polycarbonate membrane of the insert. Co-culture were conducted for 24 h in biochemical incubator with 28 °C. Fig. 1b Light microphotograph for large yellow croaker adipose tissue (AT) cells. Red arrows mark the macrophages in AT. Fig. 1c Light microphotograph for large yellow croaker liver tissue (LT) cells. Bar = 500 μm. LT, liver tissue; AT, adipose tissue. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

$\triangle\triangle^{CT}$ method) was adopted to calculate the expression of target genes, and the obtained value indicated the n-fold difference relative to the standard [21].

2.8. Western blotting

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 (Solarbio life sciences, China). Polyclonal anti-Lamin B antibodies was obtained from Santa Cruz Biotechnology (USA), whereas anti-p65 antibody was purchased from Cell Signalling Technology (USA). HRP-conjugated secondary antibodies was obtained from Golden Bridge Biotechnology

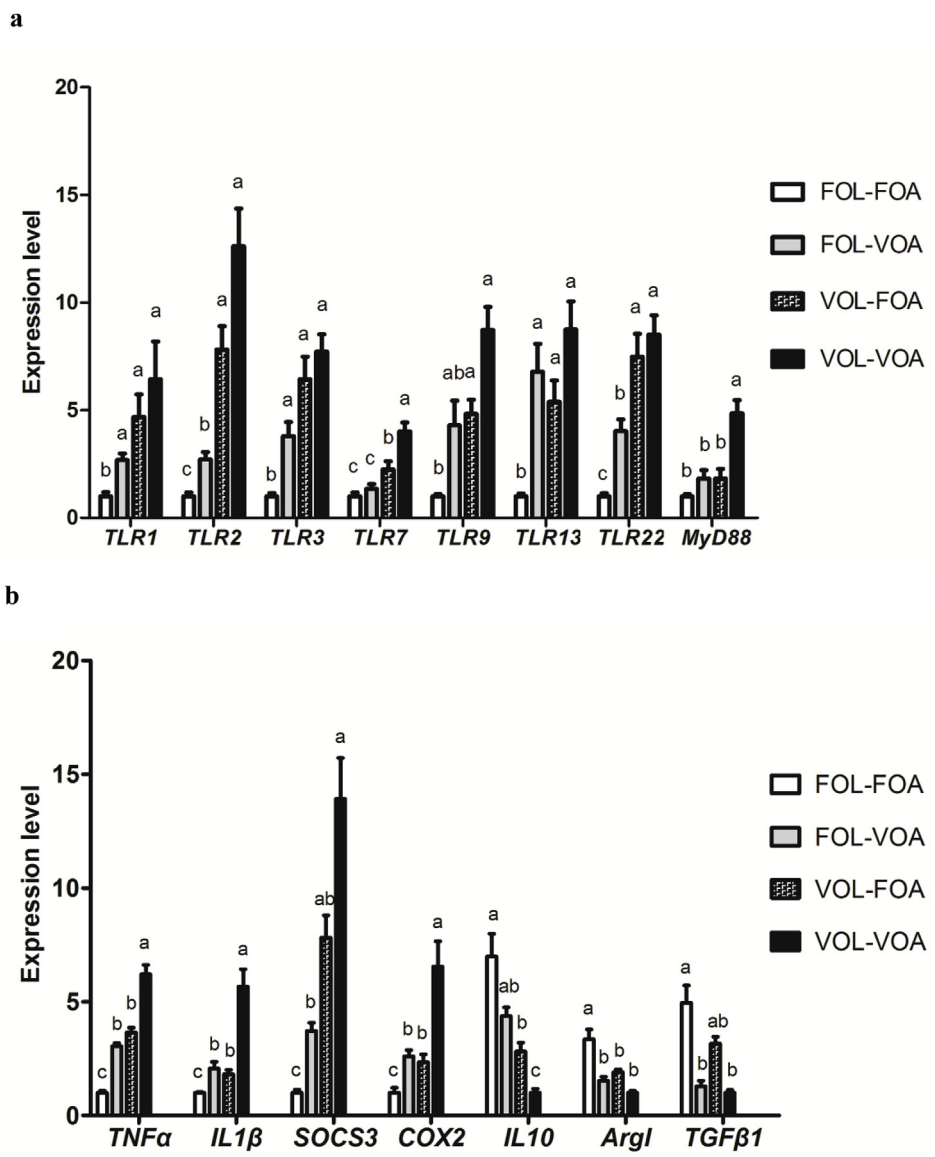

Fig. 2. RT-qPCR analyses for immune-related genes in LT cells co-cultured with AT cells. Fig. 2a TLR-related genes expression in LT cells co-cultured with AT cells. TLR-related genes expression of *TLR1*, *TLR2*, *TLR3*, *TLR7*, *TLR9*, *TLR13*, *TLR22* and *MyD88* are determined in different treatments. Fig. 2b Cytokines-related genes expression in LT cells that co-cultured with AT cells. Values are means ± S.E.M (n = 6). Different letters assigned to the bars represent significant differences using Tukey's test ($P < 0.05$). *TLR*, *toll-like receptor*; *MyD88*, *myeloid differentiation factor 88*; *IL1β*, *interleukin 1β*; *TNFα*, tumour necrosis factor α; *COX2*, *cyclooxygenase 2; SOCS3*, *suppressor of cytokine signalling 3; Arg I*, *arginase I*; *IL10*, *interleukin 10*; *TGFβ1*, *transforming growth factor β1*; FOL, liver tissue cells from fish fed FO diet; FOA, adipose tissue cells from fish fed FO diet; VOL, liver tissue cells from fish fed VO diet; VOA, adipose tissue cells from fish fed VO diet; FOL-FOA means FOL co-cultured with FOA, the control; S.E.M., stand error of the means.

(China).

2.9. Statistical analysis

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. Immune-related gene expression of LT cells

Co-culture of FOL and FOA was selected as the control group (FOL-FOA). Results indicated that, when compared with the control group, the expression of pro-inflammatory genes (*TLR1, TLR12, TLR13, TLR113, TLR122, MyD88, TNFα*, *IL1β*, *SOCS3* and *COX2*) in FOL was significantly increased in the co-culture group of FOL and VOA (FOL-VOA), while the expression of anti-inflammatory genes (*ArgI and TGFβ1*) in FOL was significantly depressed. On the contrary, a significantly depressed expression of pro-inflammatory genes (*TLR7, MyD88*, *TNFα*, *IL1β* and *COX2*) and increased expression of anti-inflammatory genes (*IL10*) in VOL was observed in the co-culture group of VOL and FOA (VOL-FOA) when compared with the co-culture group of VOL and VOA (VOL-VOA).

3.2. NF-κB signalling pathway activation of LT cells

In accordance with gene expression results, the expression of nuclear factor κB (NF-κB) signalling pathway p65 protein in nucleus of FOL increased significantly in the FOL-VOA group when compared with that of the control group, while decreased significantly in VOL in the VOL-FOA group when compared with that of the VOL-VOA group.

4. Discussion

Liver pro-inflammatory response induced by dietary VO limited the use of VO in fish diet. Studies have been conducted to elucidate the mechanism of pro-inflammatory response in the liver induced by VO, while no research focused on the role of AT in the liver inflammatory reaction. As an endocrine organ, AT was found to be closely related to immunity in mammalian studies [10], so this study was conducted to investigate the relationships between AT inflammation and liver inflammatory responses in large yellow croaker. To simulate the cross talk of AT and LT *in vivo*, a *trans*-well system (Fig. 1a) was adopted to

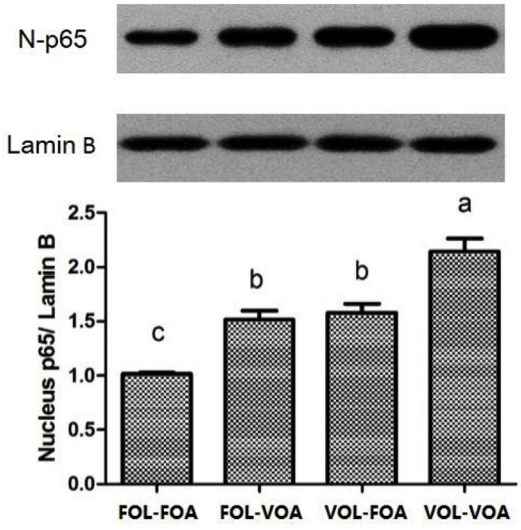

Fig. 3. Western blot analyses for NF-κB signalling. Western blot analyses NF-κB marker protein p65 of the LT cells when co-cultured with AT cells. The up panel of Fig. 3 presents the western blot image of target proteins. The down panel of Fig. 3 presents the ratio of target proteins to Lamin B. Values are means ± S.E.M (n = 6). Different letters assigned to the bars represent significant differences using Tukey's test ($P < 0.05$). NF-κB, nuclear factor κB; LT, liver tissue; AT, adipose tissue. FOL, LT cells from fish fed FO diet; FOA, AT cells from fish fed FO diet; VOL, LT cells from fish fed VO diet; VOA, AT cells from fish fed VO diet.

co-culture AT cells (Fig. 1b) and LT cells (Fig. 1c), which resulted in an assembly of the two cells types sharing the culture medium but being separated by the membrane of the insert. Small molecules, such as cytokines secreted by AT cells, could pass through the membrane.

RT-qPCR results indicated AT cells from fish fed VO diet (VOA) induced inflammation in LT cells from fish fed FO diet (FOL), manifested as the increase of pro-inflammatory gene expressions and decrease of anti-inflammatory gene expressions when compared with the control group (FOL-FOA) (Fig. 2). On the contrary, AT cells from fish fed FO diet (FOA) significantly depressed inflammation in LT cells from fish fed VO diet (VOL), when compared with that of VOL co-cultured with VOA (Fig. 2). These results indicated that large yellow croaker AT cells could affect the immune response of LT cells. The secretion of cytokines by AT cells could pass through the *trans*-well membrane and then combined with pattern recognition receptors (PRRs) on LT cell surface. PRRs transduced immune signal and activated nuclear factor κB (NF-κB) signalling pathway (Fig. 3), which could trigger the expression of downstream target genes, such as *TLRs*, *MyD88*, *IL1β*, *TNFα*, *COX2* and *SOCS3* (Fig. 2). Previous study also proved that the increase of cytokine gene expressions induced by dietary VO in AT contributed to the pro-inflammatory response of LT [3]. It could therefore be concluded that AT plays a vital role in LT pro-inflammatory response in fish fed VO diet.

Macrophages in AT are the main source of secreted cytokines [22,23]. The accumulation of macrophage in AT, called macrophage infiltration, increased the secretion of cytokines of AT [24]. Macrophage infiltration may also contribute to the pro-inflammatory response in AT induced by dietary VO, which was found to have increased the expression of macrophage infiltration marker protein in our previous study [3]. Moreover, the results of AT section in this study showed a higher amount of macrophages in AT cells from fish fed VO diet than in FO diet (S.1). The increase of macrophage infiltration may be due to the decrease of n-3 LC-PUFA content in VO diet (Table 2), because in mammalian study omega-3 fatty acids reduced the macrophage infiltration of AT and in turn decreased the pro-inflammatory response in liver [25]. However, in Atlantic salmon, when 80% of dietary FO was replaced by VO, no significant difference of macrophage accumulation was found in all dietary groups of visceral adipose tissue [26]. As interpreted by the author, these results may be due to stronger tolerance to dietary VO for Atlantic salmon, because 80% of VO does not have a significant influence on low-grade inflammation in visceral adipose tissue. Besides, AT sampled in the present study was subcutaneous adipose tissue, which was found to be more active in immunity and metabolism than visceral adipose tissue [27].

In summary, LT pro-inflammatory response induced by VO was partly due to the increase of cytokines secreted by AT, which may be attributed to the macrophage infiltration.

Author contributions statement

Qinghui Ai and Kangsen Mai designed the study. Peng Tan, Xueshan Li, Xiaojing Dong, Xiaojun Xiang and Songlin Li performed the study. Peng Tan analyzed the data and wrote the paper. All authors read and approved the final manuscript. The authors declare no competing financial interests.

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Appendix A. Supplementary data

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