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Vitamin D inhibits lipopolysaccharide-induced inflammatory response potentially through the Toll-like receptor 4 signalling pathway in the intestine and enterocytes of juvenile Jian carp (*Cyprinus carpio* var. Jian)

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Abstract

The present study was conducted to investigate the anti-inflammatory effect of vitamin D both in juvenile Jian carp (*Cyprinus carpio* var. Jian) *in vivo* and in enterocytes *in vitro*. In primary enterocytes, exposure to 10 mg lipopolysaccharide (LPS)/l increased lactate dehydrogenase activity in the culture medium ($P<0·05$) and resulted in a significant loss of cell viability ($P<0·05$). LPS exposure increased ($P<0·05$) the mRNA expression of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6 and IL-8), which was decreased by pre-treatment with 1,25-dihydroxyvitamin D (1,25D3) in a dose-dependent manner ($P<0·05$). Further results showed that pre-treatment with 1,25D3 down-regulated Toll-like receptor 4 (TLR4), myeloid differentiation primary response gene 88 (Myd88) and NF-κB p65 mRNA expression ($P<0·05$), suggesting potential mechanisms against LPS-induced inflammatory response. *In vivo*, intraperitoneal injection of LPS significantly increased TNF-α, IL-1β, IL-6 and IL-8 mRNA expression in the intestine of carp ($P<0·05$). Pre-treatment of fish with vitamin D_3 protected the fish intestine from the LPS-induced increase of TNF-α, IL-1β, IL-6 and IL-8 mainly by downregulating TLR4, Myd88 and NF-κB p65 mRNA expression ($P<0·05$). These observations suggest that vitamin D could inhibit LPS-induced inflammatory response in juvenile Jian carp *in vivo* and in enterocytes *in vitro*. The anti-inflammatory effect of vitamin D is mediated at least in part by TLR4-Myd88 signalling pathways in the intestine and enterocytes of juvenile Jian carp.

Key words: Lipopolysaccharides: Enterocytes: *Cyprinus carpio* var. Jian: Toll-like receptor 4-myeloid differentiation primary response gene 88 signalling pathway

Vitamin D is a steroid hormone, which is either synthesised from pre-vitamin D in the skin during exposure to UV light or through dietary intake in terrestrial vertebrates[1]. Vitamin D has two major forms: 25-hydroxyvitamin D (25D3) and 1,25-dihydroxyvitamin D (1,25D3). 25D3 is the major storage form of vitamin D, which is catalysed by the enzyme 1α-hydroxylase (CYP27B1) to produce the main active metabolite 1,25D3[2]. Vitamin D has been well-known for its role in bone mineralisation and Ca homoeostasis. Emerging evidence from basic research studies reveals that it also has an important role in regulating the immune system, including immune responses to bacterial infection in mammals[3–5]. Khoo *et al.*[6] reported that vitamin D_3 down-regulated pro-inflammatory cytokine production induced by *Mycobacterium tuberculosis* in peripheral blood mononuclear cells (PBMC). Zhao *et al.*[7] demonstrated that dietary vitamin D supplementation attenuates immune response of pigs challenged with rotavirus in pig. These results indicate that vitamin D mediates innate immune response. However, most research on the antibacterial action of vitamin D has been carried out in mammals, and there are limited studies in fish. To our knowledge, there is only one report on the involvement of vitamin D_3 in the modulation of the fish immune system. Cerezuela *et al.*[8] reported that diet supplementation with 0·94 mg/kg vitamin D_3 for 2 or 4 weeks resulted in a significant increase in phagocytic ability and serum peroxidase content. Physiological studies have suggested that the vitamin D_3 system in teleost is similar to that in other vertebrates[9–11]. However, the structure and form of the immune system is different between fish and mammals[12]. Whether vitamin D_3 exerts protective effects against bacterial infection in fish is unclear.

Abbreviations: 1,25D3, 1,25-dihydroxyvitamin D; DMEM, Dulbecco's modified Eagle's medium; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinases; TLR4, Toll-like receptor 4.

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The intestine is an important immune organ of fish[13,14]. The intestinal epithelial cells are the first line of defence against pathogenic bacteria present in the lumen of the gut. Besides acting as a physical barrier, epithelial cells utilise a variety of innate immune mechanisms to reduce the risk of infection from invading foreign agents, including bacterial lipopolysaccharide (LPS)[15,16]. The normal immune response of the intestine has been found to be correlated with its intestinal health in fish[17,18]. Pro-inflammatory cytokines have an important role in intestinal immunity[19]. To date, there is scarce information about the effect of vitamin D on intestinal immunity in fish. LPS is a cell wall component of gram-negative bacteria and a potent immunostimulant. Recently, LPS has been extensively used in studies of various aspects of induced immune responses in fish enterocytes[20–23]. Mulder *et al.*[22] reported that exposure to *Aeromonas salmonicida* induced the expression of *TNF-α*, *IL-1β* and *IL-8* gene in the intestine of rainbow trout. Previous studies in our laboratory also demonstrated that LPS exposure improved *TNF-α*, *IL-1β* and *IL-6* gene expression in the intestine of carp[23]. Therefore, we used LPS-induced inflammatory responses in isolated enterocytes and the intestine as a model to investigate vitamin D_3's anti-inflammatory effect in fish.

Toll-like receptor 4 (TLR4) is an important mediator of the host inflammatory response to infection. LPS induces the interaction of TLR4 with adaptor molecule myeloid differentiation primary response gene 88 (*MyD88*), which activates downstream mitogen-activated protein kinases (MAPK) and NF-κB signalling pathways and subsequently causes inflammatory cytokine production such as TNF-α, IL-1, IL-6 and IL-12[24–26]. Our previous study also demonstrated that the TLR4 signalling pathway could be activated by LPS exposure in the intestine of Jian carp[23]. Khoo *et al.*[6] reported that 1,25D3 modulated the balance in cytokine production towards an anti-inflammatory profile by repression of TLR4 expression in PMBC. Sadeghi *et al.*[27] also reported that vitamin D_3 down-regulated monocyte TLR4 expression. These facts suggest that vitamin D_3 might influence the TLR4-Myd88 signalling pathway against LPS-induced inflammatory response in the intestine of fish, which warrants investigation.

The present study was conducted to investigate the effects of vitamin D_3 on LPS-induced inflammatory responses *in vivo* and *in vitro* and to explore whether the anti-inflammatory effect is mediated through TLR4-Myd88 signalling pathways in this experiment.

Methods

Chemicals

LPS, 1,25D3, insulin, collagenase, dispase, transferrin, benzyl penicillin and streptomycin sulphate were purchased from Sigma. Hank's balanced salt solution (HBSS) and fetal bovine serum (FBS) were purchased from Hyclone. 1,25D3 stock solutions of 10^{-3} M were prepared in 100 % dimethyl sulfoxide, and further dilutions were performed using Dulbecco's modified Eagle's medium (DMEM). All 1,25D3 working solutions were stored in Eppendorf tubes at −80°C. 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) was purchased from Promega Corporation.

In vitro experiments

Primary enterocyte culture. Cell isolation and culture were performed according to the methods of Jiang *et al.*[28,29]. Briefly, healthy Jian carps (56·78 (SEM 2·8) g) were maintained for approximately 24 h without feeding before the experiment, and killed by decapitation. The intestines were rapidly separated from the carcass, opened and rinsed with HBSS-containing antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). Cells were isolated by enzymatic dissociation using collagenase and dispase, followed by physical disaggregation. Then, cells were suspended in DMEM (containing 2 % D-sorbitol, S-DMEM) and washed with S-DMEM five times to remove any undigested material and single cells according to Booth and O'Shea[30] with slight modifications. Isolated enterocytes were cultured in DMEM supplemented with 5 % FBS, 0·02 mg transferrin/ml, 0·01 mg insulin/ml and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). Cultures were kept at 26 (SEM 0·5)°C in twenty-four-well culture plates (Falcon) that had been coated with collagen I (Sigma), as previously described by Jiang *et al.*[31]. The cells were allowed to attach to plates for 72 h.

Lipopolysaccharide-induced cytotoxicity and inflammatory response in enterocytes. The cells were stimulated for 24 h with 10 mg LPS/l; this concentration was chosen because the previous experiment showed that 10 mg LPS/l of medium could induce inflammatory response in carp enterocytes[23]. Cell viability was quantified by MTS assay. Cytotoxicity was assessed by determining the release of lactate dehydrogenase (LDH) from enterocytes. The TNF-α and IL-1β mRNA expression levels were detected in cell lysates.

Prevention of lipopolysaccharide-induced inflammatory response by 1,25-dihydroxyvitamin D in enterocytes. To investigate the effect of 1,25D3 on cytokine levels in LPS-treated cells, enterocytes seeded into twenty-four-well plates were pre-treated with different concentrations of suggesting potential 1,25D3 for 72 h, and then cultured for 24 h with 10 mg LPS/l in a 27°C incubator. There were six groups (1,25D3 pre-treatment + LPS exposure): Ctrl + Ctrl (1,25D3 and LPS free), Ctrl + LPS (1,25D3 free + LPS), 1 pM-1,25D3 + LPS, 10 pM-1,25D3 + LPS, 100 pM-1,25D3 + LPS and 200 pM-1,25D3 + LPS. At the end of the experiment, media were collected to analyse LDH release. Cell lysates were collected to detect mRNA expressions of TNF-α, IL-1β, IL-6, IL-8, IL-10, TLR4, Myd88, NF-κBp65 and MAPKp38.

In vivo experiments

The Animal Care and Use Committee of Sichuan Agricultural University approved all experimental procedures.

Feeding trial. A total of 300 fish with an average initial weight of 12·58 (SEM 0·23) g from the acclimatisation aquarium were randomly assigned into two groups of three replicates, each of sixty fish. The groups were respectively fed the Ctrl diet (non-supplemented vitamin D_3) and the VD_3 diet

Table 1. Feed formulation and proximate composition of the experimental diets (air-dry basis)

Ingredients	g/kg
Soyabean meal	260
Fish meal	90
Rapeseed meal	230
Maize gluten meal	60
Rice polishings	54
Soya oil	41
Wheat middling	200
Choline chloride	6
Monocalcium phosphate	15
L-Lys·HCl	3
DL-Met	1
Bentonite	20
Vitamin premix*	10
Mineral premix†	10
Total	1000
Nutrient levels‡	
Crude protein	313
Crude lipid	67·3
Available P	6·9

* The vitamin premix provides for per kg of diet: retinyl acetate 275 mg/g, 0·80 g; cholecalciferol 12·5 mg/g, 0 g for control and 0·48 g for VD_3; DL-*α*-tocopherol acetate (50 %), 20·00 g; menadione (23 %), 0·22 g; cyanocobalamin (1 %), 0·10 g; D-biotin (2 %), 5 g; folic acid (96 %), 0·52 g; thiamine hydrochloride (90 %), 0·13 g; ascorhyl acetate (93 %), 7·16 g; niacin (99 %), 2·58 g; inositol (99 %), 52·33 g; calcium-D-pantothenate (98 %), 3·07 g; riboflavin (80 %), 0·99 g; pyridoxin (81 %), 0·75 g.

† Mineral premix provides for per kg of diet: $ZnSO_4{\cdot}7H_2O$ (22·5 % Zn), 21·64 g; $MgSO_4{\cdot}H_2O$ (15 % Mg), 230·67 g; $FeSO_4{\cdot}7H_2O$ (19·7 % Fe), 69·695 g; $CuSO_4.5H_2O$ (25 % Cu), 1·201 g; $MnSO_4$ H_2O (31·8 % Mn), 3·774 g; KI (3·8 % I), 2·895 g; $NaSeO_3$ (1 % Se), 2·50 g.

‡ Available P was calculated according to National Research Council (1993), whereas the others were measured according to the method of the Association of Official Analytical Chemists (1998).

(supplemented 0·06 mg/kg vitamin D_3) for 60 d. Experimental diets were formulated in our laboratory (Table 1). Vitamin D_3 was added in the form of cholecalciferol. For this, vitamin D_3 was first dissolved in ethanol in the appropriate doses and then dissolved in cod oil, which was sprayed on the pellets before feeding fish. The Ctrl diet was sprayed with cod oil only. Procedures for diet preparation and storage were the same as those described by Cerezuela *et al.*[8]. The experimental conditions were the same as in our previous study[32].

Lipopolysaccharide exposure trial. After a 60 d feeding trial, the fish were weighed and collected for LPS exposure trial. There were three different groups: that is, control group (Ctrl/Ctrl), LPS exposure alone group (Ctrl/LPS) and VD_3 + LPS exposure group (VD_3/LPS). There were thirty-six fish in each group, with three replicates per group and twelve fish per replicate. The fish of Ctrl/Ctrl and Ctrl/LPS groups came from the fish fed the Ctrl diet, and the fish of VD_3/LPS group were from the fish fed VD_3 diet in the feeding trial. Each fish of the Ctrl/Ctrl group was injected intraperitoneally with 100 µl of sterile PBS. Each fish of Ctrl/LPS and VD_3/LPS groups was injected intraperitoneally with 100 µl of *Escherichia coli* LPS serotype 0111:B4 (3 mg of LPS/kg of fish) diluted in sterile PBS. The LPS concentration used in this study was according to our previous study, which has been proven to induce inflammatory response[23]. After 48 h of exposure, the intestines were quickly removed, frozen in liquid nitrogen and stored at −70°C for further analysis.

Analysis and measurement

Cell viability assays. After enterocytes were stimulated for 24 h with 10 mg LPS/l, cell viability was quantified using the CellTiter 96® AQueous One Solution cell proliferation assay kit. In brief, at the time of experimental termination, 40 µl of MTS working solution was added to each well. After incubation for 2 h at 27°C in a humidified atmosphere, the amount of formazan was estimated by optical density at 490 nm on a plate reader (Wellscan MK3; Labsystems).

Lactate dehydrogenase activity measurement

LPS-induced cytotoxicity was quantified by measuring the amounts of LDH released into the culture medium from injured cells[33,34]. The amount of LDH released was measured using the method of Mulier *et al.*[35].

RNA extraction and quantitative real-time PCR analysis. The RNA extraction and quantitative real-time PCR analysis were identical to those described in our previous study[36]. Total RNA was isolated using RNAiso Plus (TaKaRa) followed by DNAse I treatment, and then 1 µg of total RNA was used to synthesise cDNA using the PrimeScript™ RT reagent Kit (TaKaRa). The RT products (cDNA) were stored at −80°C. Specific primers for the *TNF-α*, *IL-1β*, *IL-6*, *IL-8*, *IL-10*, *TLR4*, *Myd88*, *NF-κBp65* and *MAPKp38* genes were designed with Primer Premier software (Premier Biosoft International) based on the carp sequences (Table 2). The PCR mixture consisted of 1 µl of the first-strand cDNA sample, 0·5 µl of each of forward and reverse primers from 10 µM-stocks, 3 µl of RNase-free dH_2O and 5 µl of 2 × Ssofast EvaGreen Supermix (Bio-Rad). Cycling conditions were 98°C for 10 s, followed by forty cycles of 98°C for 5 s, annealing at a different temperature (Table 2) for each gene for 10 s and 72°C for 15 s. Target gene mRNA concentration was normalised to the mRNA concentration of the reference gene EF1a. After verification that the primers were amplified with an efficiency of approximately 100 %, the results were analysed using the $2^{-\Delta\Delta C_t}$ method[37]. Target and housekeeping gene amplification efficiencies were calculated according to the specific gene standard curves that were generated from 10-fold serial dilutions.

Statistical analysis

Results are presented as means with their standard errors. Data were subjected to one-way ANOVA followed by the Duncan's multiple-range test to determine significant differences among treatments using SPSS 13.0 (SPSS Inc.). A *t* test was used for comparisons between two groups. $P < 0{\cdot}05$ was considered to be statistically significant.

Table 2. Primers and annealing temperature used for in real-time quantitative PCR

Name	Sequence(5′ → 3′)	Product size	Annealing temperature (°C)	GenBank ID
TNF-α-QF	TCAACAAGTCTCAGAACA	112 bp	56	AJ311800
TNF-α-QR	GCACCTATTAAATGGATGG			
IL-1β-QF	ACAGCCTCCTCTTCTTCAG	110 bp	56·5	AJ245635
IL-1β-QR	CACCTTCTCCCAATCATCAAA			
IL-6-QF	TAGGTTAATGAGCAAGAGGA	115 bp	55·5	AY102633·1
IL-6-QR	AGAGACTGTTGATACTGGAA			
IL-8-QF	ATGAGTCTTAGAGGTCTGGGTG	114 bp	60	JN663841
IL-8-QR	ACAGTGAGGGCTAGGAGGG			
IL-10-QF	GCATACAGAGAAATACAGAACT	102 bp	55	AB110780
IL-10-QR	GTGACAGCCATAAGGACTA			
TLR4-QF	TGTCGCTTTGAGTTTGAAT	77 bp	55	HM564033
TLR4-QR	TCCAGAATGATGATGATGATG			
Myd88-QF	AAGAGGATGGTGGTAGTCA	75 bp	55·5	GU321987
Myd88-QR	GAGTGCGAACTTGGTCTG			
NF-κB p65-QF	TATTCAGTGCGTGAAGAAG	77 bp	58	LN590704
NF-κB p65-QR	TATTAAAGGGGTTGTTCTGT			
MAPKp38-QF	ACCTCAATAATATCGTCAA	159 bp	56	AB023481
MAPKp38-QR	TAAGTTCACAGTCTTCATT			
EF1α-QF	TCACCATTGACATTGCTCTC	93 bp	56	AF485331
EF1α-QR	TGTTCTTGATGAAGTCTCTGT			

MAPK, mitogen-activated protein kinase; *Myd88*, myeloid differentiation primary response gene 88; *TLR*, Toll-like receptor 4.

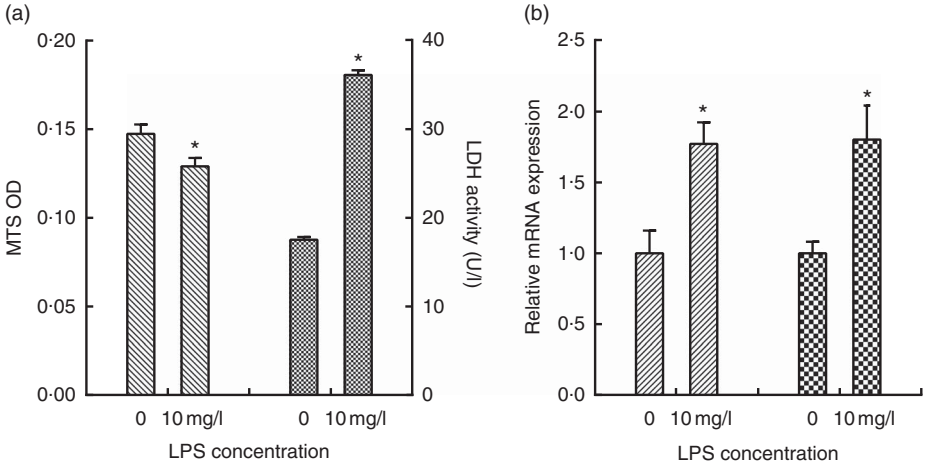

Fig. 1. 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) optical density (OD) and lactate dehydrogenase (LDH) release (a) and expression of TNF-*α* and IL-1*β* (b) in carp enterocytes in response to lipopolysaccharide (LPS) challenge. The primary cultured carp enterocytes were stimulated with 10 mg/l LPS for 24 h. Values are means (*n* 6) with their standard errors represented by vertical bars. *Mean values are significantly different ($P < 0·05$). ▨, TNF-*α*; ▩, IL-1*β*.

Results

Lipopolysaccharide-induced cytotoxicity and inflammatory response in enterocytes

To assess LPS-induced cytotoxicity in carp enterocytes, cells were incubated with 10 mg LPS/l. The cell viability and LDH activity were measured 24 h later. The result indicated that cells exposed to LPS resulted in a significant loss of viability (Fig. 1(a)). LDH release could be a good indicator of cellular damage. LPS exposure significantly increased LDH activity in medium ($P < 0·05$) (Fig. 1(a)). The expression of TNF-*α* and IL-1*β* mRNA in enterocytes with LPS treatment was measured by RT-PCR (Fig. 1(b)). The results indicated that TNF-*α* and IL-1*β* mRNA levels were significantly increased by LPS exposure compared with the unexposed group ($P < 0·05$).

Effect of 1,25-dihydroxyvitamin D on lipopolysaccharide-induced cytokine production in enterocytes

To determine whether 1,25D3 could exert an anti-inflammatory effect *in vitro*, we assessed the effect of 1,25D3 on LPS-induced inflammatory response by measuring TNF-*α*, IL-1*β*, IL-6, IL-8, and IL-10 mRNA expression in cells treated with LPS with or without 1,25D3. Cells with LPS alone resulted in significant increases in TNF-*α*, IL-1*β*, IL-6, IL-8 and IL-10 mRNA expression as compared with Ctrl/Ctrl treatment ($P < 0·05$) (Table 3). Pre-treatment of

British Journal of Nutrition

Table 3. mRNA expression of TNF-α, IL-1β, IL-6, IL-8 and IL-10 in carp enterocytes in response to 1,25-dihydroxyvitamin D (1,25D3) and lipopolysaccharide (LPS) challenge*
(Mean values with their standard errors; *n* 6)

	TNF-α		IL-1β		IL-6		IL-8		IL-10	
Groups	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Ctrl + Ctrl	1·00	0·08^{a}	1·00	0·09^{a}	1·00	0·10^{a}	1·00	0·11^{a}	1·00	0·23^{a}
Ctrl + LPS	2·54	0·15^{d}	2·12	0·33^{d}	2·14	0·10^{d}	1·70	0·14^{d}	1·90	0·14^{b}
1 pM-1,25D3 + LPS	2·45	0·17^{d}	1·97	0·21^{c,d}	2·04	0·09^{c,d}	1·50	0·08^{c,d}	1·83	0·11^{b}
10 pM-1,25D3 + LPS	1·94	0·09^{c}	1·75	0·06^{c}	1·84	0·12^{c}	1·42	0·10^{b,c,d}	1·94	0·12^{b}
100 pM-1,25D3 + LPS	1·60	0·11^{b}	1·38	0·09^{b}	1·55	0·12^{b}	1·24	0·12^{a,b,c}	2·15	0·39^{b,c}
200 pM-1,25D3 + LPS	1·33	0·22^{b}	1·17	0·13^{a,b}	1·09	0·04^{a}	1·14	0·25^{a,b}	2·33	0·33^{c}

* The cells were pre-treated with different concentrations (0, 1, 10, 100, 200 pM) of 1,25D3 for 72 h before stimulation with 10 mg/l LPS for 24 h.
^{a,b,c,d}Values with unlike letters within the same columns are statistically different ($P < 0·05$).

enterocytes with 1,25D3 inhibited the LPS-induced TNF-α, IL-1β, IL-6 and IL-8 mRNA expression in a dose-dependent manner. Treatment with 10–200 pM-1,25D3 led to a statistically significant decrease in TNF-α, IL-1β and IL-6 mRNA expression when compared with Ctrl/LPS ($P < 0·05$) (Table 3). The addition of 1,25D3 (100–200 pM) to cells significantly down-regulated IL-8 mRNA expression ($P < 0·05$) (Table 3). In contrast, the IL-10 mRNA expression was increased significantly at 200 pM-1,25D3 pre-treatment ($P < 0·05$) (Table 3).

Effects of 1,25-dihydroxyvitamin D on Toll-like receptor 4-myeloid differentiation primary response gene 88 signalling pathways in lipopolysaccharide-stimulated enterocytes

The present results have shown 1,25D3 to have anti-inflammatory effect in carp enterocytes. We determined whether the involvement of TLR4-Myd88 signalling pathways in 1,25D3-mediated inhibition of pro-inflammatory cytokine. The regulation of TLR4, Myd88, NF-κBp65 and MAPKp38 mRNA expression during LPS exposure with and without 1,25D3 treatment was investigated in carp enterocytes (Table 4). As shown, TLR4, Myd88, NF-κBp65 and MAPKp38 mRNA expression increased markedly after 24 h of stimulation with LPS ($P < 0·05$) and 1,25D3 markedly inhibited LPS-induced TLR4, Myd88 and NF-κBp65 mRNA expression ($P < 0·05$). However, the addition of 1,25D3 did not alter MAPKp38 mRNA levels ($P > 0·05$).

Vitamin D_3 decreases lipopolysaccharide-induced cytokine production in vivo

Dietary vitamin D_3 supplements administered for 60 d significantly increased the growth of carp when compared with the Ctrl group; the final weight was 48·8 (SEM 1·9) *v.* 42·1 (SEM 1·2) g ($P < 0·05$). The effects of dietary supplementation with vitamin D_3 on *TNF-α*, *IL-1β*, *IL-6*, *IL-8* and *IL-10* gene transcript abundance in the intestine of juvenile Jian carp after LPS exposure are presented in Fig. 2. The result indicated that the expression levels of the *TNF-α*, *IL-1β*, *IL-6*, *IL-8* and *IL-10* genes were increased by LPS exposure alone compared with the unexposed control group ($P < 0·05$). Vitamin D_3 pre-supplementation significantly depressed the TNF-α, IL-1β, IL-6 and IL-8 mRNA levels ($P < 0·05$). Fish exposed to LPS showed an increase in IL-10 mRNA expression of intestine as compared with the Ctrl/Ctrl group ($P < 0·05$). IL-10 mRNA expression in fish pre-feeding with vitamin D_3 was significantly up-regulated ($P < 0·05$).

The effects of dietary supplementation with vitamin D_3 on TLR4, Myd88, NF-κBp65 and MAPKp38 mRNA expression in the intestine of fish following LPS exposure are shown in Fig. 2. The results showed that, compared with Ctrl/Ctrl treatment, Ctrl/LPS caused a significant increase in TLR4, Myd88, NF-κBp65 and MAPKp38 mRNA expression levels in the intestine ($P < 0·05$). Pre-feeding with vitamin D_3 significantly prevented the up-regulation of TLR4, Myd88 and NF-κBp65 mRNA expression in the intestine. However, pre-feeding with vitamin D_3 did not

Table 4. mRNA expression of Toll-like receptor 4 (TLR4), myeloid differentiation primary response gene 88 (*Myd88*), *NF-κB p65* and mitogen-activated protein kinases (*MAPKp38*) in carp enterocytes in response to 1,25-dihydroxyvitamin D (1,25D3) and lipopolysaccharide (LPS) challenge*
(Mean values with their standard errors; *n* 6)

	TLR4		Myd88		NF-κB p65		MAPKp38	
Groups	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Ctrl + Ctrl	1·00	0·14^{a}	1·00	0·08^{a}	1·00	0·07^{a}	1·00	0·08^{a}
Ctrl + LPS	1·98	0·58^{e,f}	1·94	0·27^{c}	2·45	0·15^{d}	1·81	0·18^{b}
1 pM-1,25D3 + LPS	1·82	0·34^{d,e}	1·84	0·13^{c}	2·25	0·08^{c,d}	1·83	0·51^{b}
10 pM-1,25D3 + LPS	1·60	0·38^{c,d}	1·71	0·33^{c}	2·13	0·52^{c}	1·75	0·08^{b}
100 pM-1,25D3 + LPS	1·36	0·23^{b,c}	1·42	0·05^{b}	1·64	0·03^{b}	1·89	0·10^{b}
200 pM-1,25D3 + LPS	1·08	0·30^{a,b}	1·20	0·17^{a,b}	1·11	0·13^{a}	1·65	0·32^{b}

* The cells were pre-treated with different concentrations (0, 1, 10, 100, 200 pM) of 1,25D3 for 72 h before stimulation with 10 mg/l LPS for 24 h.
^{a,b,c,d,e,f}Values with unlike letters within the same column were statistically different ($P < 0\ 05$).

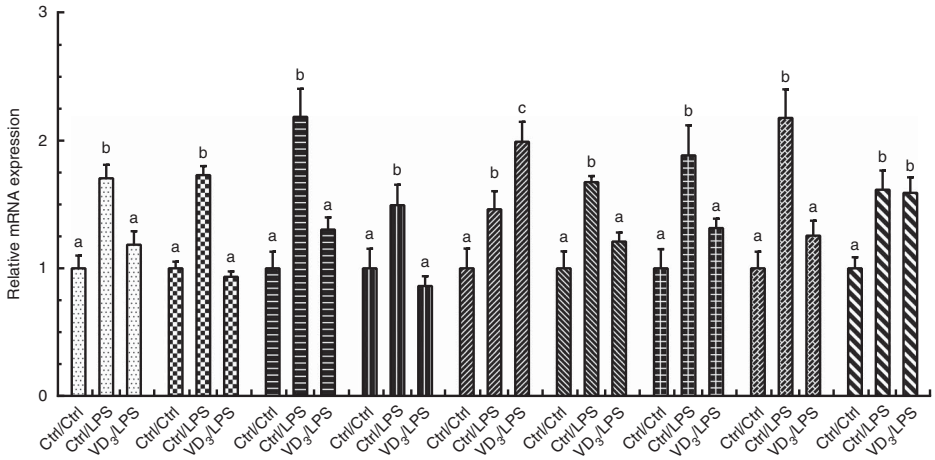

Fig. 2. Expression of TNF-*α*, IL-1*β*, IL-6, IL-8, IL-10, Toll-like receptor 4 (TLR4), myeloid differentiation primary response gene 88 (*Myd88*), *NF-κB p65* and mitogen-activated protein kinases (*MAPKp38*) mRNA in the intestine of juvenile Jian carp fed diets containing different vitamin D_3 (VD_3) levels for 60 d, followed by exposure to 3 mg lipopolysaccharide (LPS)/kg of fish for 2 d. Values are means (*n* 6) with their standard errors represented by vertical bars. ^{a,b,c}Mean values with unlike letters were significantly different ($P < 0{\cdot}05$). ⁙, TNF-*α*; ▩, IL-1*β*; ▬, IL-6; ▮, IL-8; ▨, IL-10; ▧, TLR4; ▥, MyD88; ▨, NF-κB p65; ▧, MAPKp38.

affect MAPKp38 mRNA expression compared with the results obtained from the Ctrl/LPS group.

Discussion

To our knowledge, the current study is the first evidence to demonstrate that vitamin D could inhibit LPS-induced inflammatory responses in enterocytes *in vitro* and in fish intestine *in vivo*. To study the anti-inflammatory effect of vitamin D against LPS-induced inflammatory cells, we first induced inflammatory response in carp intestinal epithelial cells. LPS is a major component of the cell wall of gram-negative bacteria implicated in the pathogenesis of bacterial infection, which is widely used as a toxicant to establish *in vitro* models of inflammatory response-induced injury in fish[23,38]. The cytotoxic effect has been assessed by markers such as cell viability and LDH release[39,40]. The present results demonstrated that exposure to LPS (10 mg/l) alone significantly increased LDH levels in the medium, indicating severe enterocyte damage. A colorimetric assay using the dye MTS can rapidly quantify the cell viability of European eel (*Anguilla anguilla* L.) PBMC[41,42]. Using this assay, the present study showed that cell viability was depressed by LPS exposure. This result was in good agreement with our previous report[23]. Cytokines, such as TNF-*α*, IL-1*β*, IL-6 and IL-8, have a fundamental role in the regulation of the pro-inflammatory response in fish, having a pivotal role throughout the infection process[21]. In the current study, the exposure of carp enterocytes to LPS caused a significant increase in IL-1*β*, TNF-*α*, IL-6 and IL-8 mRNA levels, indicating a stimulatory action upon pro-inflammatory processes. Therefore, to induce inflammatory response in carp enterocytes, cells were incubated for 24 h with 10 mg LPS/l.

In fish, protection of the digestive tract against pathogenic attack is crucial for maintaining health, as a large number of pathogenic microorganisms invade through its surface[43]. TNF-*α* and IL-1*β* are two of the most important pro-inflammatory cytokines; their inappropriate expression or overexpression can lead to the progression of inflammatory and autoimmune diseases[44]. As a principal cytokine, IL-1*β* is a strong regulator of the expression of other cytokines, such as IL-6 and IL-8[45,46]. The present result clearly demonstrated that

the expression levels of TNF-α, IL-1β, IL-6 and IL-8 were up-regulated in enterocytes in response to LPS exposure. 1,25D3 pre-treatment markedly inhibited LPS-induced up-regulation of TNF-α, IL-1β, IL-6 and IL-8 mRNA levels in enterocytes. This may indicate that 1,25D3 has a potential role in the inhibition of intestinal inflammation induced by LPS. Previously, 1,25D3 was demonstrated to inhibit pro-inflammatory cytokines in human corneal epithelial cells colonised with *Pseudomonas aeruginosa*[47]. Our results are consistent with these reports. IL-10 is a pleiotropic cytokine with significant anti-inflammatory properties, which is a key regulator in the maintenance of immunological homoeostasis[48]. The present study showed that 1,25D3 treatment significantly up-regulated the expression of IL-10 mRNA. These results suggest that 1,25D3 could attenuate the intestinal inflammatory response in fish. To date, no study has been conducted to investigate the effect of 1,25D3 on the inflammatory cytokines gene expression in the intestine of fish.

TLR4 is a member of the TLR family of pattern recognition receptors that specifically mediates signalling by LPS. Classically, TLR4 recognises the microbial lipids in homodimer format, and thus activates various intracellular signalling pathways, such as the NF-κB and MAPK pathways[49,50]. Recently, a TLR4 sequence has been identified experimentally in Chinese rare minnow, and even two *TLR4* genes were found in the zebra fish genome[51,52]. MacKenzie and Milston reported that teleost fish also display LPS responsiveness[53–55]. Su *et al.*[51] demonstrated that the TLR4 signalling pathway can be triggered by grass carp *reovirus* and *Aeromonas hydrophila* infection in rare minnow. Thus, it is possible that piscine *TLR4* gene was already implicated in LPS sensing. To clarify the cellular mechanisms that regulated the cytokine production after LPS exposure, we examined the effect of 1,25D3 pre-treatment of carp enterocytes on LPS-induced MyD88-dependent signalling. The MAPKp38 and NF-κBp65 are the family members of MAPK and NF-κB, respectively, and they are the main signalling molecules in the TLR4-Myd88 signalling pathway of their family[56–58]. Over-activation of this signalling pathway would aggravate inflammatory reaction exacerbating their negative effects on the fish. Our data indicate that LPS exposure up-regulated the expression of TLR4, Myd88, MAPKp38 and NF-κBp65 mRNA in enterocytes. Pre-treatment with 1,25D3 inhibited the up-regulation of TLR4, Myd88 and NF-κBp65 mRNA levels. Interestingly, 1,25D3 pre-treatment did not alter MAPKp38 mRNA expression. Studies in various cell types, including dendritic cells[59–61], pancreatic islet cells[62] and kidney cells[63], indicated that vitamin D dampens NF-κB signalling. Our observations are in accordance with those reports, but how vitamin D_3 interacts with the TLR4 signalling pathway is unknown. However, several mechanisms have been proposed, including a vitamin D-induced increase in the levels of IκBα[64], interference with the binding of NF-κB subunits to promoter regulatory areas[61] or both.

On the basis of the beneficial effects of 1,25D3 against LPS-induced inflammatory response in the enterocytes, it was reasonable to hypothesise that vitamin D can protect fish against LPS-induced inflammatory responses *in vivo*. The present study showed that inflammation induced by intraperitoneal injection of 3 mg LPS/kg fish was associated with increased expression of TNF-α, IL-1β, IL-6 and IL-8 mRNAs in the intestine. A previous study has looked at the effects of LPS on the immune system in fish and has demonstrated a high potential for mediating pro-inflammatory cytokine mRNA abundance[65]. Vitamin D_3 pre-supplementation decreased TLR4, Myd88 and NF-κBp65 mRNA expression. The results presented suggest that impaired inflammatory response to LPS in fish is, at least in part, because of TLR4 down-regulation. As TLR4 is a key component in pathogen (LPS) recognition and crucial mediators in the early inflammatory response to foreign microorganisms, down-regulation of TLR4 by vitamin D_3 clearly represents an important and novel immune-modulating effect. This result was in agreement with this statement *in vitro*. Studies from monocytes also indicated that vitamin D_3 downregulates TLR4 expression and triggers hyporesponsiveness to pathogen-associated molecular patterns[27]. However, the mechanisms await further characterisation.

In conclusion, our present study demonstrated that LPS exposure could induce inflammatory response, resulting in up-regulation of TNF-α, IL-1β, IL-6 and IL-8 mRNA abundance in the intestine and in the enterocytes of fish. Dietary and medium pre-supplementation with vitamin D_3 could inhibit LPS-induced immune damage in fish intestine and the enterocytes, respectively. The anti-inflammatory effect of vitamin D_3 may associate with decreasing the expression of pro-inflammatory cytokines by downregulating TLR4, Myd88 and NF-κBp65 mRNA abundance.

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J. J. and D. S. conducted the trial, performed the RT-PCR experiments, and wrote the manuscript. Y. Z. and X. Z. contributed to the design of the study. L. F. and W. J. assisted in the manuscript preparation. Y. L. and L. T. assisted with all data analysis. L. Y. and P. W. assisted with the trail.

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British Journal of Nutrition

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