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Nutrient sensing signaling functions as the sensor and regulator of immunometabolic changes in grass carp during *Flavobacterium columnare* infection

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ABSTRACT

In order to illustrate the immunometabolic changes of fish during bacterial infection, grass carp (*Ctenopharyngodon idellus)* was injected with *Flavobacterium columnare (F. columnare)* and then the immune response, nutrient metabolism and related signaling pathways were assayed from 6 h post injection (hpi) to 7 days post injection (dpi). After *F. columnare* injection, gill lamellae showed obvious fusion and higher mRNA expression levels of pro-inflammatory cytokines. The mRNA expression levels of *TNF-α, IL-1β* and *IL-8* in the head kidney were also significantly upregulated at 6 hpi and 3 dpi. Moreover, the expression of *IgZ* in the gill was significantly upregulated at 3 dpi and 7 dpi, while the expression of *IgM* in the head kidney was significantly upregulated at 1 dpi and 3 dpi after *F. columnare* injection. During bacterial infection, the systematic nutrient metabolism was also significantly affected. Hepatic glycolysis, indicated by GK mRNA expression and PK activity, was significantly upregulated at 1 dpi, while glucogenesis, indicated by PEPCK mRNA expression and enzyme activity, was significantly increased at later time, which resulted in the decreased hepatic glycogen content at 1dpi but increased glycogen content at 7 dpi in the experimental group. LPL, which catalyzed the lipid catabolism, showed decreased mRNA expression and enzyme activity at 6 hpi, while ACC, which was rate-limiting of FA synthesis, was significantly increased at 6 hpi, 3 dpi and 7 dpi. During this process, the nutrient sensing signaling was also significantly affected. TOR signaling in grass carp was significantly activated while ERK signaling was significantly inhibited after *F. columnare* infection, both of which might function as the sensor and regulator of fish immunometabolic changes.

1. Introduction

Fish diseases induced by bacterial infection still remain the largest threat to the development of aquaculture [1]. *Flavobacterium columnare* (*F. columnare*), the casusative agent of columnaris disease, infects many freshwater fish species and results in a high degree of mortality [2]. Plenty of studies have been performed to elucidate the pathogenesis of columnaris disease and the interactions between *F. columnare* and the host [3]. Following *F. columnare* infection, the fish gills exhibited increased mucous cells [4], congestion of gill lamellae and inflammatory cell infiltration in the advanced stage [5], and fusion of gill lamellae and extensive internal hemorrhage in the more advanced stages [4]. *F. columnare* can secrete toxins to damage the tissue or enhance invasive processes and the chondroitin AC lyase, a connective-tissue-degrading enzyme, is alleged to play a role in the virulence of *F. columnare* [6]. On the other side, fish activate its own immune system when encountering an infection, including both the innate and adaptive immune system [7]. The surface mucous layer of fish has been assumed as the first physical-immunological barrier as it plays an important adhesive role in defending against external bacteria and toxicants [8]. Not only the inflammatory cell infiltration in the gill lamellae could be detected under light microscopic examination [9], the expression levels of many pro-inflammatory cytokines both in the gill and head kidney were also elevated after infection [10,11]. Early study demonstrated that the classical, antibody-mediated complement pathway of the fish immune system is highly effective in killing *F. columnare* [12]. However, *F. columnare* might be able to avoid parts of the immune system, as it might triggers the endogenous programmed cell death machinery of immune cells including apoptosis to evade the immune system [13].

The preventive measures to control columnaris disease are rather important, including management, vaccination and probiotics [14]. Furthermore, developing specific antibodies would be one potential

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with huge prospects [15]. During the early stages of the disease, antimicrobial agents and chemicals have been used to treat disease with varied success rates [2], however, the usage of these agent or chemicals might induce the risk of drug residues in fish fillet [16]. Nutritional modulation methods to improve fish immunity own their advantage and some additives have been shown to promote the immunity and disease resistance ability of fish [17]. For example, β-glucan administration induces metabolic changes and differential survival rates after bacterial or viral infection in turbot (*Psetta maxima*) [18]. Before the application of nutritional modulation, one question should be answered, as how fish modulate their own nutrient metabolism during pathogenic infection. A study in mosquito fish (*Gambusia holbrooki*) demonstrated that LPS-challenged fish could increase their production of ATP to compensate physiologically for the energetic requirements of immune functioning [19]. Metabolic changes in white shrimp (*Litopenaeus vannamei*) after white spot syndrome virus (WSSV) infection were shown to resemble the Warburg effect with increased glucose consumption and increased glucose-6-phosphate dehydrogenase (G6PDH) activity [20]. Previous studies also showed that fish metabolism was significantly affected after *F. columnare* infection. In the channel catfish (*Ictalurus Punctatus*) that was infected by *F. columnare* via immersion, the mRNA expression levels of genes involved in the carbohydrate metabolism and lipid metabolism, such as G6PCa1, lipoyl synthase, and fatty acid-binding protein, were significantly affected [21]. Another study in topmouth culter (*Culter alburnus*) also found that the differentially expressed genes after the injection of *F. columnare* were mainly associated with pathways such as immune response, carbohydrate metabolism, amino acid metabolism and lipid metabolism [22].

In fact, the crosstalk between the immune system and metabolism is an emerging interesting field of study both in human health and animal agriculture [23]. Malnutrition leads to a decrease in immune cell number, for example, both total T cell and CD4$^+$ T cell numbers from spleens of fasted mice were decreased by 40–50% compared to fed control animals [24]. On the other hand, bacterial infection can disrupt metabolic homeostasis of the hosts and induce multiple shifts in tissue metabolism involved in energy supply and demand [25]. Moreover, the shifts in energy supply and demand led to the transcriptional and translational changes, which were orchestrated by multiple metabolic sensors to regulate the intrinsic metabolic programs [26]. Among all metabolic sensors, mechanistic target of rapamycin (mTOR) and mitogen-activated protein kinase (MAPK) were shown to act as immunometabolic switches to regulate the local immune and metabolic phenotypes [27]. mTOR signaling pathway will be activated when nutrients are in abundance [28], and when immune cells are in metabolically demanding situations, such as stimulation with growth factors, nutrient availability, and immune regulatory signals [29]. Phosphorylated mTOR senses cues from the immune microenvironment, suppresses autophagy, and elevates immune cell growth and proliferation [30]. Following infection, the pathogen recognition receptors (PRRs) of the host could also activate MAPKs, which would further induce the mRNA expression levels of multiple genes that regulate immune response [31]. On the other hand, pathogenic bacteria also evolved mechanisms to directly modulate MAPK activation to inhibit the immune response [32]. For example, *S. enterica* can activate ERK1 and ERK2 via the *Salmonella* Pathogenicity Island-2 T3SS effector SteC, but it can also inactivate ERK1 and ERK2 via the phosphothreonine lyase activity of the *Salmonella* Pathogenicity Island-2 T3SS effector SpvC [33].

Till now, little information is known about the metabolic changes of fish with columnaris disease induced by *F. columnare* infection, and no information is available about the regulatory mechanism of fish immunometabolism during infection. Such information will provide possible nutritional modulatory methods to control columnaris disease. Thus, the immune response, nutrient metabolism and several nutrient sensing signalings of grass carp during *F. columnare* infection were assayed in the present study.

2. Materials and methods

2.1. Bacterial culture

F. columnare G4 strain used in the present study was obtained from the Laboratory of Aquatic Medicine of College of Fisheries, Huazhong Agricultural University (Hubei, China). *F. columnare* G4 strain was taken out from −80 °C refrigerator and recovered on plate medium at 28 °C for 24 h. Then a single colony was chosen and inoculated in modified Shieh (MS) broth at 28 °C in a shaker incubator at 100 rpm for 24 h to reach the final OD540 at 0.6. The colony forming units per milliliter (CFU/mL) in the final culture were measured by plate counting under a microscope.

2.2. Fish husbandry and bacterial infection

Juvenile grass carp (mean ± SEM: 40.0 ± 5.0 g; mixed sex) were bought from a fish farm in Wuhan (Hubei, China) and maintained in six independent circular fiberglass tanks (80 cm in dimeter, 100 cm in column height), with the water turnover rate of 10.6 L min^{-1}. All tanks were maintained at 16 ± 2 °C, under a 12:12 light:dark photoperiod. Fish were fed twice per day with a commercial diet (38% crude protein and 4% crude lipid) from Wuhan Zhengda Aquatic Products Co. Ltd (Hubei, China).

In the experimental group, fish were intraperitoneally injected with *F. columnare* at a final concentration of 2×10^6 CFU/mL, after anesthesia with MS222 (1:10000). Sham-challenge (control group) followed an identical procedure using MS broth inoculum without bacteria for another three tanks of fish. After challenge, fish were returned to the previous tanks with the same water conditions and kept starved till the end of the experiment. Fish samples were obtained from two groups at 6 h post injection (hpi), 1 day, 3 days, and 7 days post injection (dpi). Four individuals were sampled from each tank. All sampled fish were firstly anesthetized using MS222 (1: 10000) and killed. Then gill, head kidney, liver and other tissues were taken out. Partial segments of the gill were stored in paraformaldehyde. Other tissue samples (including gill, liver, head kidney and other tissues) were immediately frozen in liquid nitrogen and then stored at −80 °C until analysis for nucleotide extraction and Western blot analysis.

2.3. Histological observation of tissue section

The gill samples of grass carp were removed, fixed with paraformaldehyde and then transferred into 70% ethanol after 24 h. Then, about 0.5-cm length segments of distal intestinal were transversely sliced into 5-μm sections and then stained with Alcian Blue (A.B.) staining kit (Solarbio, China). The slides were examined under a light microscope (Olympus, DP72) equipped with a camera (Nikon E600) and CellSens Standard Software (Olympus) for image acquisition.

2.4. DNA and RNA extraction, cDNA synthesis and quantitative RT-PCR

Genomic DNA was extracted from tissue samples (liver, muscle, head kidney, swim bladder, gut, gill and gall bladder) of grass carp using the Universal Genomic DNA Kit (CwBiotech, China) following the manufacturer's instructions, which were then used to amplify a 1460 base pair fragment flanking *F. columnare* gene with a specific primer set (Table 1). RNA was extracted from the gill, liver and head kidney using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendations. The integrity and purity of RNA were checked with 1% agarose gel electrophoresis and Nanodrop 2000 spectrophotometer (Thermo scientific, USA), respectively. In order to remove the potential genomic DNA, the RNA samples were treated with DNase, then 1 μg of the resulting total RNA was reverse transcribed into cDNA using the Prime Script Reverse Transcriptase kit (Takara) according to the manufacturers' instructions.

Table 1
Primer used in the present study.

	GenBank No.	Forward Sequence	Reverse Sequence
F. columnare 16S		GCCCAGAGAAATTTGGAT	TGCGATTACTAGCGAATCC
TNFα	HQ696609	CATCCATTTAACAGGTGCATAC	GCAGCAGATGTGGAAAGAGAC
IL-1β	JN705663.2	TCCTCGTCTGCTGGGTGT	CAAGACCAGGTGAGGGGAAG
IL-8	JN663841	CATGTCTGACCATTACTGAAGC	GTTTCCTTCAGGGTGGCAATG
P65 NF-*κB*	KJ526214	GAAGAAGGATGTGGGAGATG	TGTTGTCGTAGATGGGCTGAG
IkBα	KJ125069	TCTTGCCATTATTCACGAGG G	TGTTACCACAGTCATCCACCA
Ikkα	KM279718	GGCTACGCCAAAGACCTG	CGGACCTCGCCATTCATA
IgM	DQ417927	GCTGAGGCATCGGAGGCACAT	TTGGGTCTCGCACCATTTTCTC
IgD	GQ429174	CTGGCGCAGCTCTGAATTTG	TCGGAGGATGCTCACAATGG
IgZ	GQ201421	GGGGTACCATGCAAAAAAAGTGACAG	CGGAATTCGCATGCATAATCTTTGTTACA
pIgR		ACCCGCAGTACATCAGCC	ATCGTCACCCTTCCCTTG
GK	ADD52460	GAAGAGCGAGGCTGGAAGG	CAGAATGCCCTTATCCAAATCC
PK	JQ951928	GCCGAGAAAGTCTTCATCGCACAG	CGTCCAGAACCGCATTAGCCAC
G6Pase	ESTs	AAAGACAGCAGGTAGAAGAGG	ACGGAAAACAAGAAGAGCAG
PEPCK	JQ898294	ATCGTCACGGAGAACCAA	CCTGAACACCAAACTTAGCA
FAS	HM802556	GATGGGTCTACAGCCTGATGG	GACACCCTGTGGACATTGAGC
LPL	FJ436077	TACAGCGGCGTTCACACTTG	CTACATGAGCACCAAGACTG
CPT-1α	JF728839	GCCACTGTAAAGGAGAACC	GGATGCCTCATAAGTCAAG
ACC	GU908475	TGGCTGCACTGCACTCTCACT	GGTCCAGCTTCCCTGCGGTC
PPAR-α	FJ231987	AGCAGAGAAGGACGTCAG	TTCCTTCTCGGCATGCTG
SREBP-1	GU339498	TACAGCCAGGCAGGAAACTCA	AGTATTCCCCATCCCCGTCA
β-Actin	M25013	GGCTGTGCTGTCCCTGTA	GGGCATAACCCTCGTAGAT

Specific primer pairs were designed using Primer Premier 5, whose amplification efficiencies were controlled at 100 ± 5%. Quantitative real-time PCR was conducted on 7500 Real-time PCR system (Applied Biosystems, USA) using the Eva Green 2 × qPCR Master mix (ABM, Canada). All samples were performed in triplicate wells and the cycling conditions were 30 s at 95 °C, 1 s at 95 °C and 10 s at 58 °C for 40 cycles. In addition, the specificity of each primer pair was verified by dissociation curve to ensure only one specific-sized single amplicon was amplified. The relative quantification of the target genes was determined via normalized against β-actin. Then relative abundance of target genes was calculated by using the $2^{-\Delta\Delta Ct}$ method [34]. All primers used in the present study are shown in Table 1.

2.5. Western blot analysis

Protein was extracted from liver tissues after homogenation with RIPA buffer (added with proteinase inhibitor and phosphorylated-protease inhibitor). The protein concentration within each sample was calculated with an enhanced BCA Protein Assay Kit (P0010, Beyotime Tech., China) and then protein concentration of all samples was adjusted to the same level. Same levels of protein from each group were separated on SDS-PAGE. Proteins were then transferred to PVDF membranes (Pall Corporation) for Western blot analysis. Primary antibody for phospho-S6 (Ser 235) was purchased from Cell Signaling (U.S.A.). Primary antibody for phospho-ERK1/2 (Thr 202/204, Thr185/187) and β-actin was purchased from ABClonal Biotechnology (China). These antibodies have been used in many fish species including turbot [35,36], rainbow trout [37,38] and grass carp [34].

2.6. Statistics

All statistical analyses were performed using SPSS 17.0. All results were tested for normality using the Shapiro Wilk's-W's test. Normally distributed data were analyzed by Factorial (two-way) analysis of variance (ANOVA) to determine the main effects of period (from 6 h to 7 days) and group (control group v. s. experimental group), and their interactions on gene expression, enzyme activity, metabolites content and protein concentration. When significant interaction of period and group were observed, data were analyzed by one-way ANOVA followed by Tukey's multiple range tests to inspect differences among all the treatments. When only a significant interaction and significant main effects of period or group were observed, data were analyzed by one-way ANOVA followed by Tukey's multiple range tests to inspect differences among periods within each group and vice versa. When the significance is only with the main effects of period or group, the data were analyzed by the two-way ANOVA followed by Tukey's multiple range tests to assess the main effects of period or group only. Differences were considered significant when $P < 0.05$. All data were expressed as mean ± standard deviation of the mean (SD).

2.7. Key resources table

Resource	Source
Antibodies	
phospho-ERK1/2	ABClonal Biotechnology
phospho-S6	Cell Signaling Technology
ß-actin	ABClonal Biotechnology
Chemical	
Alcian Blue staining kit	Solarbio Technology
Universal Genomic DNA kit	Cw Biotechnology
Prime Script Reverse Transcriptase	Takara
Eva Green 2*qPCR Master Mix	ABM
BCA protein assay kit	Beyotime Technnology

3. Results

3.1. *F. columnare* infection seriously affected gill morphological structure

PCR amplifications with most DNA samples in the experimental group were positive for *F. columnare,* while PCR results with samples in the control group were negative, which indicated the successful infection (Fig. 1). Then gill histological structures were evaluated. Lesions were observed in gill after *F. columnare* injection, with exfoliation of respiratory epithelia cells from the infected gill. The secondary lamellae (SL) length of the gill in the experimental group was much shorter than its length in the control group (Fig. 2). The affected gill histological structures after *F. columnare* infection significantly affected their physiological capacity.

3.2. *F. columnare* infection significantly induced inflammation in the gill and head kidney

Pro-inflammatory cytokines expression, including *IL-1β* and *IL-8,* in the gill of the experimental group was significantly upregulated at 1dpi

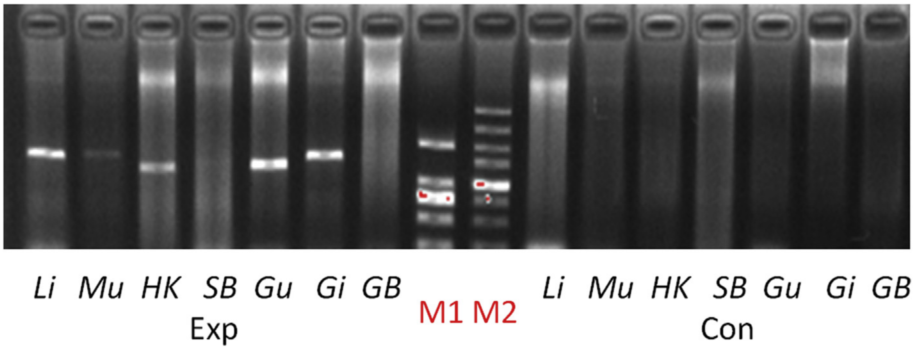

Fig. 1. PCR results of DNA samples from grass carp tissues to amplify a 1460 base pair fragment flanking *F. columnare* gene. Li: liver; Mu: muscle; HK: head kidney; SB: swim bladder: Gu: gut; Gi: gill; GB: gall bladder. M1: DNA Marker 1500bp; M2: DNA Marker 2500bp. Con: Control group; Exp: Experimental group.

and 3 dpi, with the highest expression level detected at 1 dpi (Fig. 3a). Even at 7 dpi, *IL-1β* expression in the gill of the experimental group was still significantly higher than in the gill of the control group. However, *TNF-α* expression in the gill of the experimental group was only upregulated at 7 dpi. The expression of genes involved in NF-κB signaling was also significantly affected in the gill after infection. *IκKα* expression in the gill of the experimental group was significantly upregulated from 1dpi to 7 dpi, and highest expression level was also detected at 1 dpi. *IκBα* expression in the gill of the experimental group was only upregulated at 7 hpi, and *p*65 NF-*κB* expression in the experimental group was also significantly higher than in the control group.

All these pro-inflammatory cytokines expression in the head kidney of the experimental group were also significantly upregulated from 1 dpi to 3 dpi, with the highest expression level detected at 3 dpi (Fig. 3b). Similar trend was also found in the expression of *IκKα and IκBα*, whose highest expression level were also detected at 1dpi. *p*65 NF-*κB* expression in the head kidney of the experimental group was also significantly upregulated at 1 dpi and 3 dpi.

3.3. *F. columnare infection significantly induced up-regulation of Igs in the gill and head kidney*

As shown in Fig. 4a, *IgZ* expression in the gill of the experimental group was significantly upregulated at 3 dpi and 7 dpi. *IgM* expression in the gill of the control and experimental group showed not significant difference, but significant differences were detected among different periods. *IgD* and *poly immunoglobulin receptor* (*pIgR)* expression in the gill of the experimental group was significantly upregulated at 7 dpi.

IgM expression in the head kidney of the experimental group was significantly upregulated at 1 dpi and 3 dpi (Fig. 4b). No significant difference in the *IgZ* expression was detected between the head kidney of the control and experimental group, but *IgZ* expression in the head

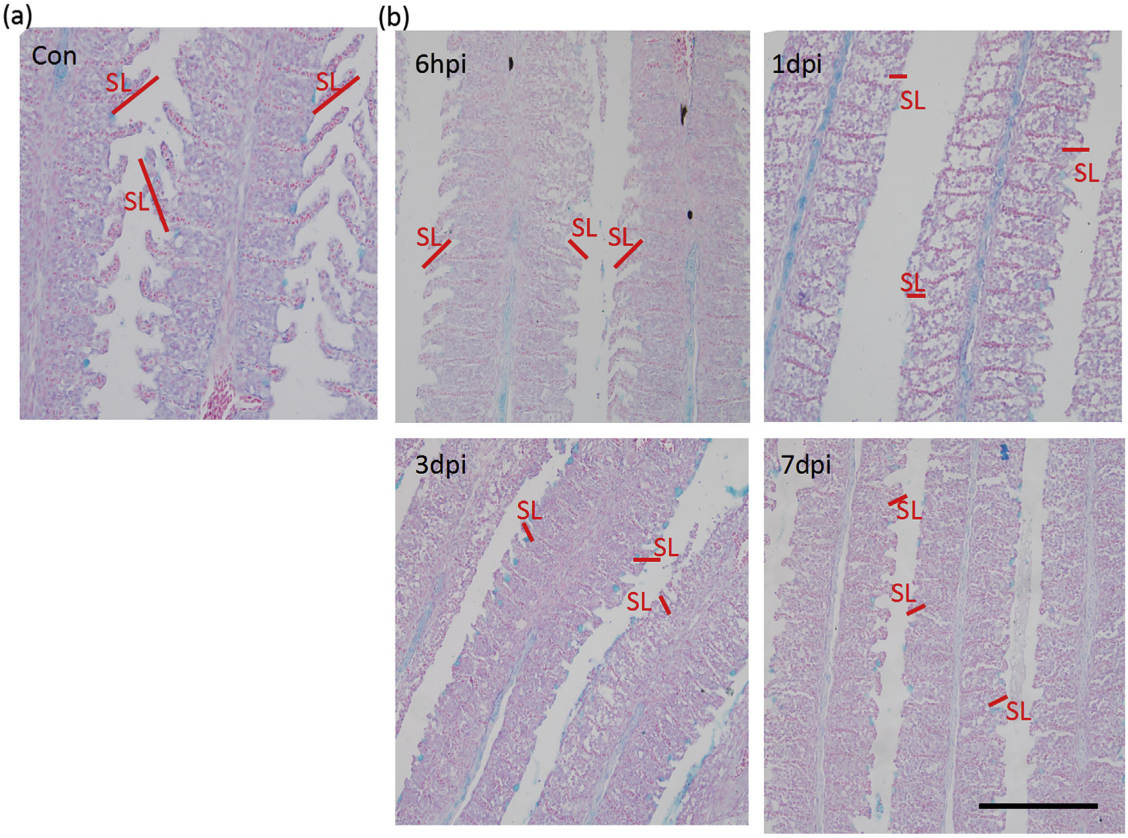

Fig. 2. The histological structures in the gill of grass carp in the control group (a) and experimental group at 6 h, 1 d, 3 d, and 7 d (b) after *F. columnare* injection. All sections were stained with A.B. staining. SL: secondary lamellae. Scale bars, 50 μm.

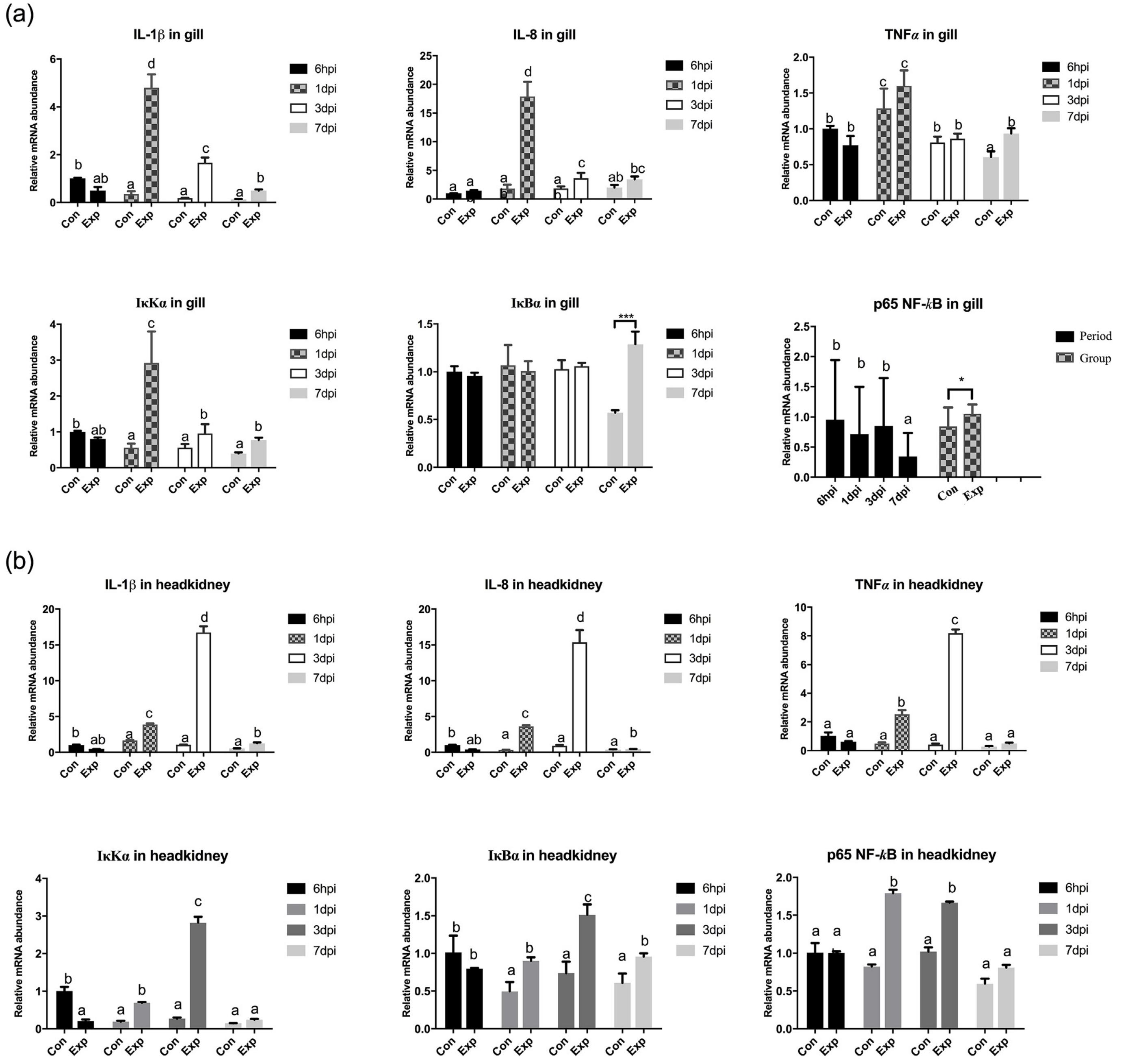

Fig. 3. The relative mRNA expression levels of pro-inflammatory cytokines (*TNF-α, IL-1β, IL-8*) and genes involved in NF-κB pathway (*NF-κB, Ikkα, IkBα*) in the gill (a) and head kidney (b) of grass carp at different periods (6 h, 1 d, 3 d and 7 d) after injection of *F. columnare* (Exp) or MS broth (Con). Statistical analysis was performed by two-way ANOVA. Data are means ± SD (n = 3). Mean values with different letters indicated significant difference among groups, $P < 0.05$. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

kidney showed variation among different periods. *IgD* expression in the head kidney of the experimental group was significantly downregulated at 7 dpi, while no significant differences were found in the *pIgR* expression among all periods in two groups.

3.4. *F. columnare infection significantly affected signaling pathways involved in immune response and nutrient metabolism*

As shown in Fig. 5, *F. columnare* infection resulted in the activation of TOR signaling and the inactivation of ERK signaling. The activation of TOR signaling, indicated by p-S6/β-actin protein level, were significantly higher in the experimental group than in the control group at all periods, with highest level detected at 3 dpi. The inactivation of ERK signaling, indicated by p-ERK1/2/β-actin protein level, was detected in the experimental group at 6 hpi, 1 dpi and 7 dpi.

3.5. *F. columnare infection significantly affected systemic nutrient metabolism*

GK expression in the liver of the experimental group, which catalyzed the glycolysis, was significantly upregulated from 1 dpi to 7 dpi (Fig. 6a). Another gene involved in glycolysis, PK, also exhibited increased mRNA expression level in the liver of the experimental group at 1 dpi and 7 dpi. Hepatic PK enzyme activity in the experimental group was also significantly upregulated at 1 dpi. On the other hand, PEPCK expression in the liver of the experimental group, which was involved

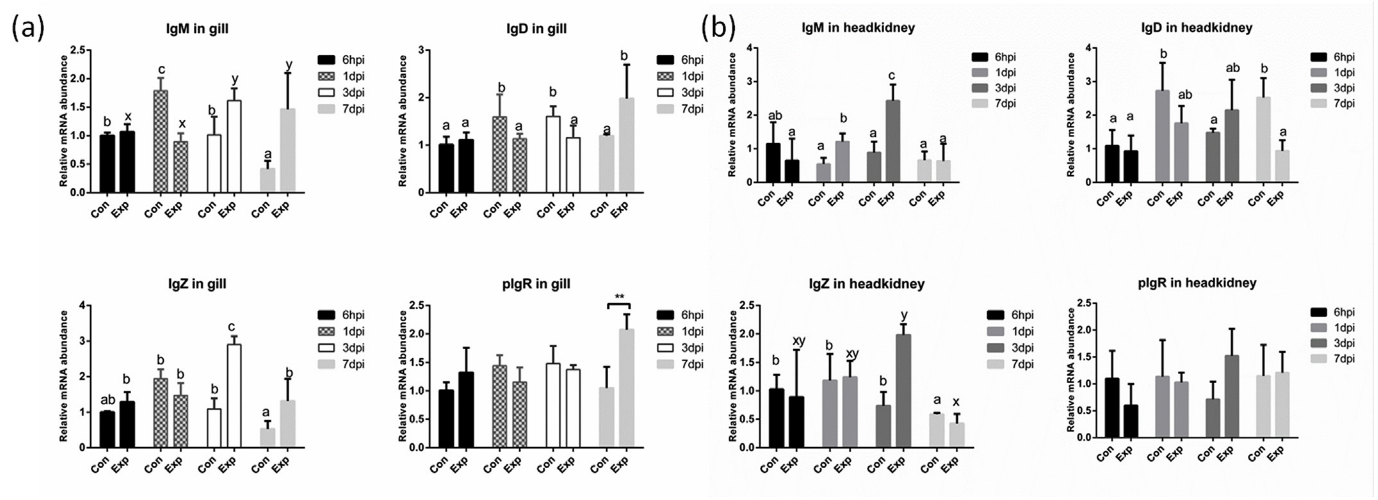

Fig. 4. The relative mRNA expression levels of immunoglobulins (*IgM, IgD, IgZ*) and their receptor (*pIgR*) in the gill (a) and head kidney (b) of grass carp at different periods (6 h, 1 d, 3 d and 7 d) after injection of *F. columnare* (Exp) or MS broth (Con). Statistical analysis was performed by two-way ANOVA. Data are means ± SD (n = 3). Mean values with different letters indicated significant difference among groups, $P < 0.05$. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

in gluconeogenesis, was significantly upregulated at 7 dpi (Fig. 6b). Hepatic PEPCK enzyme activity in the experimental group was significantly upregulated at 1dpi and 3 dpi. G6Pase, which was also involved in gluconeogenesis, was not significantly affected by *F. columnare* infection, but showed decreased expression level with increased period. The glycogen content in the liver of the experimental group was also significantly affected, which significantly decreased at 1dpi but increased at 7 dpi in the experimental group (Fig. 6c).

LPL expression in the liver of the experimental group, which catalyzed the lipid catabolism, was significantly decreased at 6 hpi and 3 dpi (Fig. 7a). Similar trend was also found in the LPL enzyme activity, which was significantly decreased in the experimental group at 6 hpi. *Cpt-1α* expression in the liver of the experimental group was significantly increased at 7 dpi. Similarly, PPAR*α* expression in the liver of the experimental group was significantly decreased at early period (1 dpi), but increased at later period (7 dpi). On the other hand, ACC expression in the liver of the experimental group, which catalyzed the FA synthesis, was significantly upregulated at 6 hpi, 3 dpi and 7 dpi (Fig. 7b). FAS, another gene involved in FA synthesis, was not significantly affected by *F. columnare* infection, but showed decreased expression level with increased period. The total triglyceride (TG) content in the liver was not significantly affected by *F. columnare* infection, but significantly decreased with increased period in two groups.

4. Discussion

In the present study, grass carp was infected with *F. columnare*, and then the immune response, nutrient metabolism and related signaling pathway were evaluated. Among all the previous experimental infection trials with *F. columnare*, two inoculation routes were mainly adopted, i.e. bath (immersion) and injection [3]. The water temperature during the experimental period was lower than 20 °C, resulting in the low virulence of bacteria [39,40]. Former studies have shown that injection of low virulent bacteria more readily induced infection and disease contraction than direct surface contact [41,42]. So in the

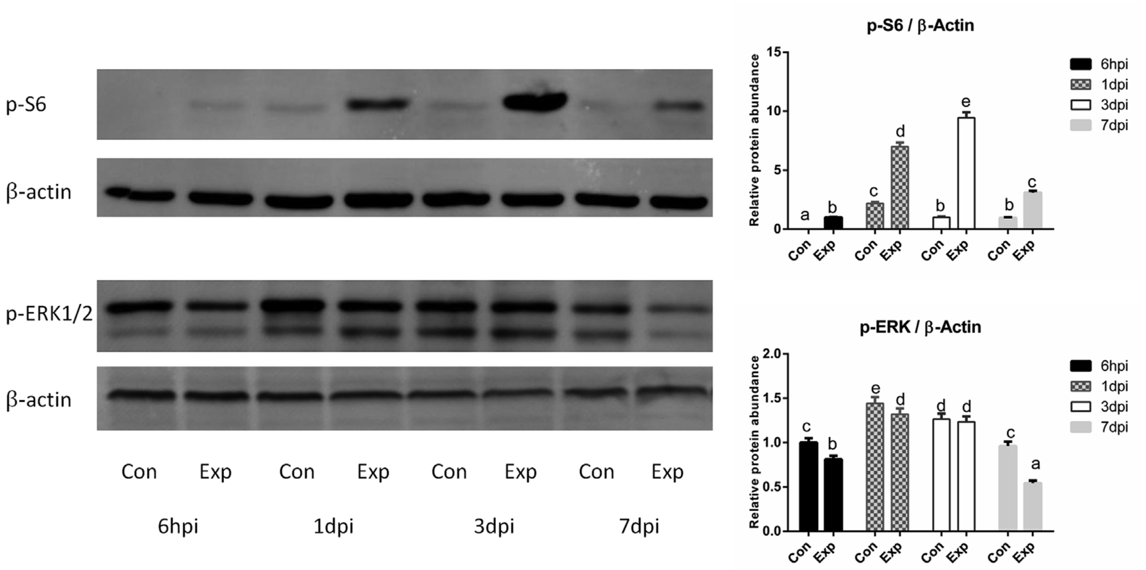

Fig. 5. The relative protein abundance of p-S6, p-ERK1/2 and *β*–actin in the grass carp at different periods (6 h, 1 d, 3 d and 7 d) after injection of *F. columnare* (Exp) or MS broth (Con). Statistical analysis was performed by two-way ANOVA. Data are means ± SD (n = 3). Mean values with different letters indicated significant difference among groups, $P < 0.05$.

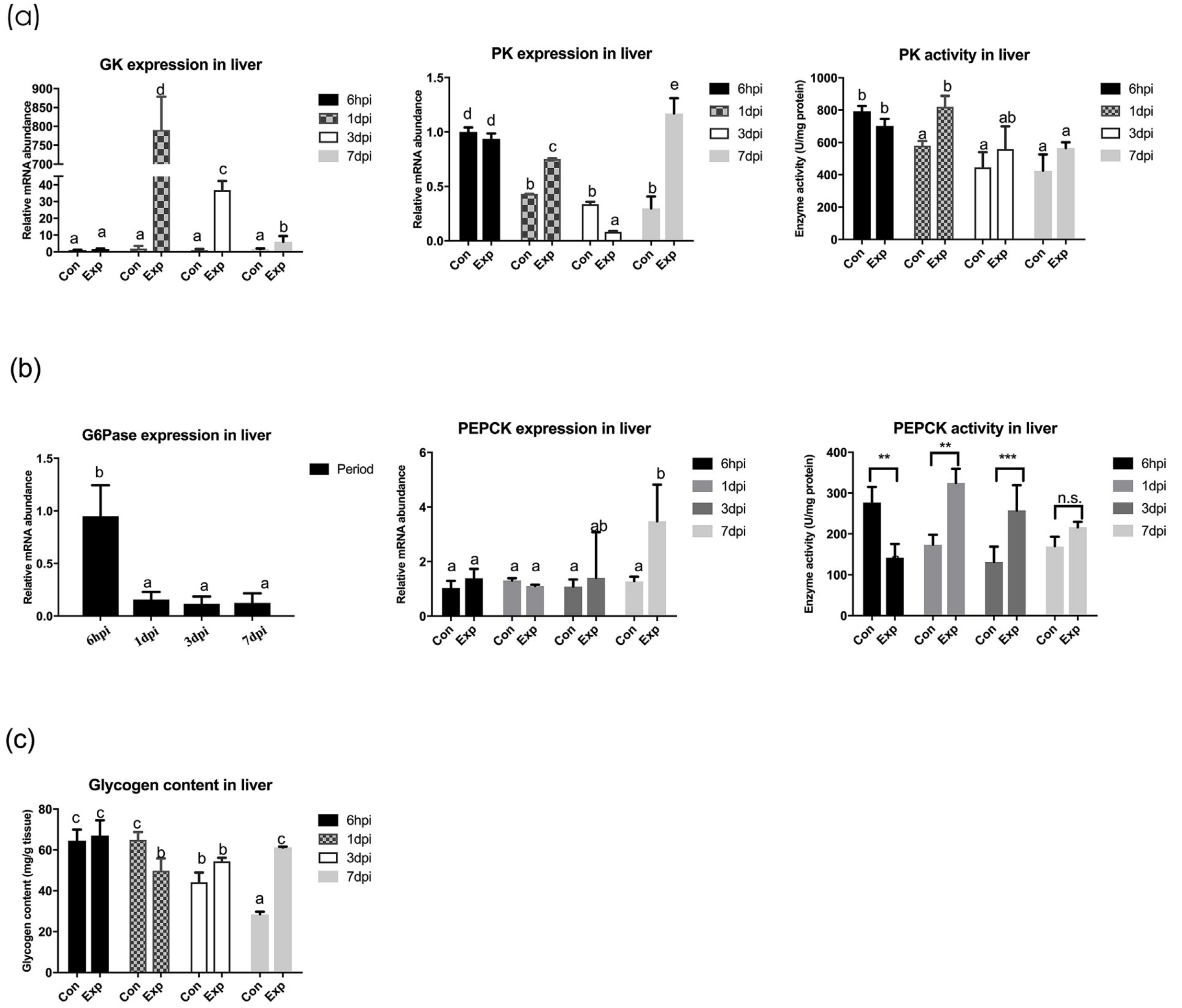

Fig. 6. The hepatic carbohydrate metabolism of grass carp at different periods (6 h, 1 d, 3 d and 7 d) after injection of *F. columnare* (Exp) or MS broth (Con). (a) The mRNA expression levels of GK and PK, and PK enzyme activities in liver. (b) The mRNA expression levels of G6Pase and PEPCK, and PEPCK enzyme activities in liver. (c) The glycogen content in liver. Statistical analysis was performed by two-way ANOVA. Data are means ± SD (n = 3). Mean values with different letters indicated significant difference among groups, $P < 0.05$. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

present study, grass carp was infected with *F. columnare* via intraperitoneal injection. PCR results of DNA samples from liver, muscle, head kidney, gut, and gill of the experimental group were all positive, confirming the successful *F. columnare* infection in grass carp. Moreover, the gill morphology in the experimental group was significantly affected, as the gill lamellae got thickening and the length of the secondary lamellae (SL) was much smaller than control fish. Obvious hyperplasia of epithelial cells was detected in the gill of the experimental group, which was similar to earlier studies in yellow catfish [43] and pond loach [44]. The intact structure of mucosal tissues functions as the first physiological barrier against pathogen attack, and the serious lesion of gill mucosal surfaces would result in the activated immune response.

During pathogenic infection, several specific pattern rocognization receptors (PRRs) within the immune cells were devoted to detection of pathogen-associated molecular patterns (PAMPs) [45]. After the activation of PRRs signaling pathway, several major effector cells would be recruited into the site of infection for the induction for inflammation and the activation of the adaptive immune response [46,47]. In the present study, the mRNA expression of cytokines, including *IL-1β, IL-8* and *TNF-α,* in the gill and head kidney was significantly upregulated after *F. columnare* infection, which is similar to earlier studies in mandarin fish [48] and channel catfish [10]. Moreover, studies in topmouth culter (*Culter alburnus*) [24], mandarin fish [11,48], and grass carp [49] reported that the cytokines expression in the head kidney after *F. columnare* infection was upregulated from 36 hpi to 7 dpi. Other studies in channel catfish [10,21] reported that the cytokines expression in the gill was significantly increased at earlier time from 1 h to several hours post infection. Rather few studies evaluated the influence of *F. columnare* infection on the cytokines expression level within mucosal tissues and systematic tissues at the same time. Our previous study in pond loach found that the expression of *IL-15* and *IL15R-α* was upregulated at much earlier time in the gill and skin (3 hpi and 12 hpi) than in the head kidney and spleen (12 hpi and 1dpi) [50]. In the present study, the highest expression of cytokines in the gill was detected at 1 dpi, while the highest expression of cytokines in the head kidney mainly was detected at 3 dpi. So the upregulation of pro-inflammatory cytokines after bacterial infection seems to occur much earlier in gill than in head kidney. Moreover, NF-κB signaling was reported to be involved in the regulation of inflammation [51,52]. In the present study, *IκKα* expression showed the similar trend like the above-mentioned cytokines, as the highest *IκKα* expression in the gill was detected at 1 dpi in the experimental group, while the highest *IκKα* expression in the head kidney was detected at 3 dpi in the experimental group. This is similar to previous reports in catfish, as the expression of *IL17R-A* and *NF-κB* in the gill after *F. columnare* immersion showed a trend to increase at early

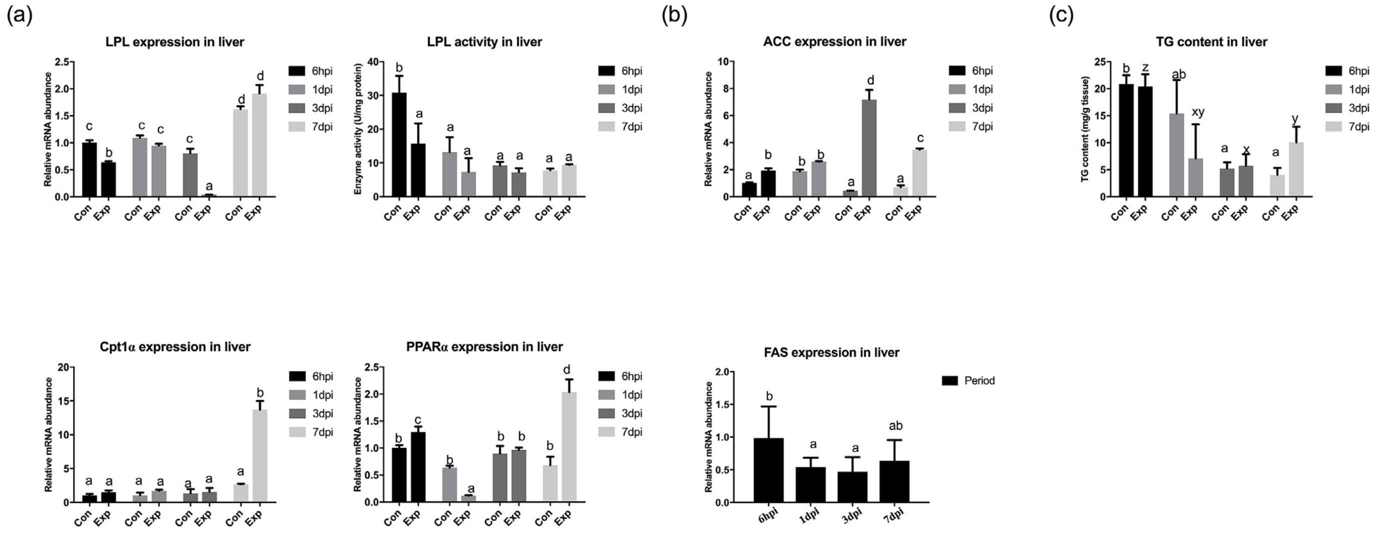

Fig. 7. The hepatic lipid metabolism of grass carp at different periods (6 h, 1 d, 3 d and 7 d) after injection of *F. columnare* (Exp) or MS broth (Con). (a) The mRNA expression levels of *LPL, CPT-1α* and *PPAR-α,* and LPL enzyme activities in liver; (b) The mRNA expression levels of *ACC* and *FAS* in liver; (c) The triglyceride (TG) content in liver. Statistical analysis was performed by two-way ANOVA. Data are means ± SD (n = 3). Mean values with different letters indicated significant difference among groups, $P < 0.05$.

time (4 h) and then to decrease with prolonged infection period (24 h and 48 h) [51].

Like in mammals, immunoglobulins (Igs) in teleost also play important roles in adaptive immunity to defend against extracellular pathogens attack and there exist three different immunoglobulin types in teleost, i.e. IgM, IgZ/IgT and IgD [53]. A former study in grass carp after vaccination with the recombinant fusion protein of *F. columnare* showed that both the mRNA expression level and the antibody titers of IgM were significantly higher at 3 and 4 weeks post vaccination [54]. In mandarin fish injected with inactivated *F. columnare*, the expression of *IgZ* was also upregulated in the gill at much earlier time (1 week) compared to its upregulation in the head kidney at 3 weeks [48]. Former studies have shown that IgM play a crucial role in systematic immunity while IgZ/IgT play a crucial role in mucosal immune response including gut [55], gill [56], skin [53]. In the present study, *IgM* expression level was upregulated at 1 dpi and 3 dpi in the head kidney, while *IgZ* expression level was upregulated at 3 dpi and 7 dpi in the gill of the experimental group. However, the expression levels of *IgM* in the gill and *IgZ* in the head kidney were not significantly affected by *F. columnare* infection, which only showed variation among different injection periods in two groups. Our results might confirm that IgZ play a more important role in mucosal tissues such as the gill, while IgM play a more important role in systemic immune organs such as the head kidney.

During pathogen attack, the activation of lymphocytes and other leukocytes initiates a program of cell growth, proliferation, and differentiation that increase metabolic demand [57]. Leukocytes do not store glycogen in a large amount, which makes them highly dependent on the import of extracellular glucose, glutamine and ATP to meet their increased metabolic needs [58]. Former studies have checked the influence of *F. columnare* infection on nutrient metabolism, for example, the differently expressed genes in *F. columnare* infected topmouch culter were mainly associated with pathways such as immune response, carbohydrate metabolism, amino acid metabolism, and lipid metabolism [22]. In the channel catfish infected with *F. columnare*, the metabolism in the gills was also significantly affected. The expression of *Apolipoprotein A-1b, Apolipoprotein C*, and *lipoyl synthase* was significantly upregulated at 1 hpi and 2 hpi [21]. Considering the fact that bacterial infection would seriously affect fish feed intake and that mRNA expression levels of nutrient metabolic genes are impacted by nutritional status, fish in two groups were kept starved after injection in order to mimic the side effects. Results showed that two hepatic genes involved in glycolysis, *GK* and *PK*, were significantly upregulated in the experimental group at 1 dpi. Moreover, PK enzyme activity was also significantly increased in the experimental group at 1 dpi, which resulted in the decreased glycogen content in the liver of the experimental group at 1 dpi. However, PEPCK, which is involved in glucogenesis, showed increased mRNA expression at 1 dpi and 3 dpi, and increased enzyme activity at 7 dpi. All these regulation contributed to the increased glycogen content in the liver of experimental group at 7 dpi. Besides carbohydrate metabolism, the lipid metabolism was also significantly affected by *F. columnare* infection. Both the mRNA expression and enzyme activity of LPL, which was involved in the lipid catabolism, were significantly decreased at 6 hpi. This is similar to early studies in rodents, as endotoxin (LPS) could induce the decreased heaptic LPL activity [59]. The expression of another gene involved in lipid catabolism, *Cpt1α,* was significantly upregulated in the experimental group at 7 dpi. Consistent with these results, an important regulating factor, *peroxisome proliferators-activated receptor alpha* (*PPARα)* [60], significantly decreased at 1 dpi, but increased at 7 dpi. The mRNA expression of *acetyl CoA carboxylase* (*ACC*), which was involved in FA synthesis, was significantly upregulated at 6 hpi, 3 dpi and 7 dpi, while FAS expression only decreased with increased period. The results suggested that metabolic reprogramming might occur in grass carp liver after *F. columnare* infection. After *F. columnare* infection, energy in grass carp might come mainly from glycolysis at early period (before 3 dpi), but from lipid catabolism with prolonged period (7 dpi).

Above results suggested that fish reprogrammed their metabolic status to supply energy for the continuous immune response to *F. columnare* infection, however, how the signal was transducted and how this modulation was regulated remains unknown. mTOR is a serine/threonine kinase that plays a role in cell growth and metabolism by sensing environmental cues, including when nutrients are in abundance and when immune cells are in metabolically demanding situations, such as stimulation with growth factors, nutrient availability, and immune regulatory signals [28]. Phosphorylated mTOR senses cues from the immune microenvironment and elevates immune cell growth and proliferation [30]. Our former studies have indicated that rapamycin treatment in fish successfully inhibited mTOR signaling [35] and mTOR signaling could sense the unbalanced amino acid level during fishmeal replacement by plant protein sources [36]. Moreover, mTOR signaling integrates nutrient metabolism and intestinal immunity in grass carp after prolonged starvation [34]. In the present study, mTOR signaling pathway in the grass carp was significantly activated during *F. columnare* infection, indicated by the increased phosphoraltion of S6. Former studies have also shown that mTOR signaling was activated during pathogenic infection [61,62]. For example, hepatitis C virus induced the mTOR/S6K axis in mammalian hepatocytes by inhibiting the TSC1/TSC2 complex [61], and human papillomavirus virus 16 was shown to induce mammalian TOR (mTOR) through upregulation of growth factor receptor signaling in human keratinocytes [62]. Infection with protozoan parasites also impacts mTOR signaling, as *Toxoplasma gondii* maintained mTOR-dependent cellular growth and phosphorylation of the ribosomal S6 protein in infected Peritoneal exudate macrophages [63]. However, in some other studies, TOR signaling was inhibited during infection. In human epithelial cells infected with *Shigella* or *Salmonella*, two enteric pathogens that invade host cells, mTOR activity is rapidly blunted [64]. In the case of *Salmonella*, this stress response is only transient and associated with the damage to the *Salmonella*-containing vacuole (SCV) [65]. So the modulation of TOR signaling by pathogenic infection might be pathogen-species specific and might also be regulated differentially at different time post infection. Besides TOR, the MAPK pathway is also involved in the infection of host cells by bacterial pathogens [66] and intracellular pathogens manipulate MAPK pathways to increase their virulence [67]. Several bacterial pathogens, including *Mycobacterium tuberculosis*, activate MAPK pathways and promote invasion of host cells [68]. In contrast, *Yersinia* sp. and *Shigella* sp. inhibit phosphorylation of MAPK and negatively regulate proinflammatory responses [69,70]. One study in trophoblast giant (TG) cells showed ERK1/2 was dephosphorylated at 0.5–6 h after *L. monocytogenes* infection [71]. In the present study, the activation of p-ERK1/2 signaling was significantly inhibited during *F. columnare* infection.

In all, the immune response of grass carp during *F. columnare* infection was accompanied with hepatic metabolic reprogramming, during which process several signaling pathways including TOR signaling and ERK signaling were involved.

List of abbreviations

ACC	acetyl-CoA carboxylase
CPT-1α	carnitine palmitoyltransferase 1 alpha
ERK1/2	extracellular regulated protein kinase 1/2
FAS	fatty acid synthase
GK	glucose kinase
G6Pase	glucose 6 phosphatase alpha
Ig	immunoglobulin
IκBα	inhibitor of nuclear factor kappa-B kinase alpha
IκKα	IκB kinase α
IL-1β	interleukin-1β
IL-8	interleukin-8
LPL	lipoprotein lipase
MS222	3-aminobenzoic acid ethyl ester
p65 NF-κB	p65 nuclear factor kappa beta
PEPCK	phosphor-enol pyruvate carboxy kinase
pIgR	poly immunoglobulin receptor
PK	pyruvate kinase
PPARα	peroxisome proliferators-activated receptor alpha
TNF-α	tumor necrosis factor-alpha

Conflicts of interest

The authors declare no conflict of interest.

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References

[1] H.D. Rodger, Fish Disease Causing Economic Impact in Global Aquaculture, Fish Vaccines, Springer, 2016.
[2] G.L. Bullock, T.C. Hsu, E.B. Shotts, Columnaris disease of fishes, USFWS Fish Dis. Leaflet (1986) 1–9.
[3] A.M. Declercq, F. Haesebrouck, W.V. den Broeck, P. Bossier, A. Decostere, Columnaris disease in fish: a review with emphasis on bacterium-host interactions, Vet. Res. 44 (2013) 27.
[4] R. Foscarini, Induction and development of bacterial gill disease in the eel (*Anguilla japonica*) experimentally infected with Flexibacter columnaris: pathological changes in the gill vascular structure and in cardiac performance, Aquaculture 78 (1989) 1–20.
[5] H.W. Ferguson, Systemic Pathology of Fish, second ed., Scottian Press, London, 2006.
[6] H.M.T. Kunttu, E.I. Jokinen, E.T. Valtonen, S.L.-R., Virulent and nonvirulent *Flavobacterium columnare* colony morphologies: characterization of chondroitin AC lyase activity and adhesion to polystyrene, J. Appl. Microbiol. 111 (2011) 1319–1326.
[7] S.K. Whyte, The innate immune response of finfish–a review of current knowledge, Fish Shellfish Immunol. 23 (2007) 1127–1151.
[8] O.F. Oscar, S.A. Bullard, M.E. Andrew, L. Maria Jose, C.R. Arias, Adhesion dynamics of *Flavobacterium columnare* to channel catfish *Ictalurus punctatus* and zebrafish *Danio rerio* after immersion challenge, Dis. Aquat. Org. 96 (2011) 221–227.
[9] H.W. Ferguson, Systemic Pathology of Fish, second ed., Scottian Press, London, 2006.
[10] P. Eric, L. Chao, B.C. Peterson, D.L. Straus, B.D. Farmer, B.H. Beck, Basal polarization of the mucosal compartment in *Flavobacterium columnare* susceptible and resistant channel catfish (*Ictalurus punctatus*), Mol. Immunol. 56 (2013) 317–327.
[11] L.W. Gai, C.W. Ming, W.Z. Xiao, X.C. Ming, X.X. Hai, P. Nie, Molecular cloning, biological effect, and tissue distribution of interleukin-8 protein in Mandarin fish (*Siniperca chuasti*) upon *Flavobacterium columnare* infection, Fish Shellfish Immunol. 66 (2017) 112.
[12] D.D. Ourth, L.M. Bachinski, Bacterial sialic acid modulates activation of the alternative complement pathway of channel catfish (*Ictalurus punctatus*), Dev. Comp. Immunol. 11 (1987) 551–564.
[13] D.V. Ana, M. Fernanda, M.T. Silva, Apoptosis of sea bass (*Dicentrarchus labrax* L.) neutrophils and macrophages induced by experimental infection with *Photobacterium damselae* subsp. piscicida, Fish Shellfish Immunol. 15 (2003) 129–144.
[14] D.L. Merrifield, A. Dimitroglou, A. Foey, S.J. Davies, R.T.M. Baker, J. Bøgwald, M. Castex, E. Ringø, The current status and future focus of probiotic and prebiotic applications for salmonids, Aquaculture 302 (2010) 1–18.
[15] J. Bebak, M. Matthews, C. Shoemaker, Survival of vaccinated, feed-trained largemouth bass fry (*Micropterus salmoides floridanus*) during natural exposure to Flavobacterium columnare, Vaccine 27 (2009) 4297–4301.
[16] J. Romero, C.G. Feijoo, P. Navarrete, Antibiotics in Aquaculture–Use, Abuse and Alternatives, (2012).
[17] V. Kiron, Fish immune system and its nutritional modulation for preventive health care, Anim. Feed Sci. Technol. 173 (2012) 111–133.
[18] L.P. Marta, M.M. Costa, F. Antonio, N. Beatriz, β-glucan administration induces metabolic changes and differential survival rates after bacterial or viral infection in turbot (*Scophthalmus maximus*), Fish Shellfish Immunol. 82 (2018) 173–182.
[19] C. Bonneaud, R.S. Wilson, F. Seebacher, Immune-challenged fish up-regulate their metabolic scope to support locomotion, PLoS One 11 (2016).
[20] C. I-Tung, A. Takashi, H. Yun-Tzu, H. Ikuo, C. Tsan-Chi, H. Jiun-Yan, C. Geen-Dong, L. Chu-Fang, W. Han-Ching, White spot syndrome virus induces metabolic changes resembling the warburg effect in shrimp hemocytes in the early stage of infection, J. Virol. 85 (2011) 12919–12928.
[21] D. Zhang, W. Thongda, C. Li, H. Zhao, B.H. Beck, H. Mohammed, C.R. Arias, E. Peatman, More than just antibodies: protective mechanisms of a mucosal vaccine against fish pathogen *Flavobacterium columnare*, Fish Shellfish Immunol. 71 (2017) 160–170.
[22] L. Zhao, J. Tu, Y. Zhang, J. Wang, L. Yang, W. Wang, Z. Wu, Q. Meng, L. Lin, Transcriptomic analysis of the head kidney of Topmouth culter (*Culter alburnus*) infected with *Flavobacterium columnare* with an emphasis on phagosome pathway, Fish Shellfish Immunol. 57 (2016) 413–418.
[23] M. Diane, S.E. Shoelson, Immunometabolism: an emerging frontier, Nat. Rev. Immunol. 11 (2011) 81.
[24] D.C. Saucillo, V.A. Gerriets, S. John, J.C. Rathmell, N.J. Maciver, Leptin metabolically licenses T cells for activation to link nutrition and immunity, J. Immunol. 192 (2014) 136–144.
[25] W. Eisenreich, J. Heesemann, T. Rudel, W. Goebel, Metabolic host responses to infection by intracellular bacterial pathogens, Front. Cell. Infect. Microbiol. 3 (2013) 24.
[26] G.K.S. Hotamisligil, E. Ebru, Nutrient sensing and inflammation in metabolic diseases, Nat. Rev. Immunol. 8 (2008) 923–934.

[27] M.H. Kogut, K.J. Genovese, H. He, R.J. Arsenault, AMPK and mTOR: sensors and regulators of immunometabolic changes during Salmonella infection in the chicken, Poult. Sci. 95 (2015) 345.
[28] M. Laplante, D. Sabatini, mTOR signaling in growth control and disease, Cell 149 (2012) 274–293.
[29] S.P. Cobbold, The mTOR pathway and integrating immune regulation, Immunology 140 (2013) 391–398.
[30] J.D. Powell, K.N. Pollizzi, E.B. Heikamp, M.R. Horton, Regulation of immune responses by mTOR, Annu. Rev. Immunol. 30 (2016) 39–68.
[31] C.J. Caunt, S.M. Keyse, Dual-specificity MAP kinase phosphatases (MKPs): shaping the outcome of MAP kinase signalling, FEBS J. 280 (2013) 489–504.
[32] C.R. Roy, E.S. Mocarski, Pathogen subversion of cell-intrinsic innate immunity, Nat. Immunol. 8 (2007) 1179–1187.
[33] C. Odendall, N. Rolhion, A. Förster, J. Poh, D.J. Lamont, M. Liu, P.S. Freemont, A.D. Catling, D.W. Holden, The Salmonella kinase SteC targets the MAP kinase MEK to regulate the host actin cytoskeleton, Cell Host Microbe 12 (2012) 657–668.
[34] S. Li J, Li Y. Zhao, Q. Zhang, Q. Wang, Nutrient sensing signaling integrates nutrient metabolism and intestinal immunity in grass carp, *Ctenopharyngodon idellus* after prolonged starvation, Fish Shellfish Immunol. 71 (2017) 50–57.
[35] Q. Wang, G. He, K. Mai, W. Xu, H. Zhou, X. Wang, L. Mei, Chronic rapamycin treatment on the nutrient utilization and metabolism of juvenile turbot (*Psetta maxima*), Sci. Rep. 6 (2016) 28068.
[36] Q. Wang, G. He, K. Mai, W. Xu, H. Zhou, Fishmeal replacement by mixed plant proteins and maggot meal on growth performance, target of rapamycin signalling and metabolism in juvenile turbot (*Scophthalmus maximus* L.), Aquacult. Nutr. 22 (2016) 752–758.
[37] W. Dai, S. Panserat, S. Kaushik, F. Terrier, E. Plagnes-Juan, I. Seiliez, S. Skiba-Cassy, Hepatic fatty acid biosynthesis is more responsive to protein than carbohydrate in rainbow trout during acute stimulations, Am. J. Physiol. Regul. Integr. Comp. Physiol. 310 (2016) R74–R86.
[38] I. Seiliez, J. Gabillard, M. Riflade, B. Sadoul, K. Dias, J. Avérous, S. Tesseraud, S. Skiba, S. Panserat, Amino acids downregulate the expression of several autophagy-related genes in rainbow trout myoblasts, Autophagy 8 (2012) 364–375.
[39] R.A. Holt, J.E. Sanders, J.L. Zinn, J.L. Fryer, K.S. Pilcher, Relation of water temperature to Flexibacter columnaris infection in steelhead trout (*Salmo gairdneri*) coho (*Oncorhynchus kisutch*), and chinook (*O. tshawytscha*) salmon, J. Fish. Res. Board Can. 32 (1975) 1553–1559.
[40] S.L.-R., M.A. Tiirola, E.T. Valtonen, Influence of rearing conditions on Flavobacterium columnare infection of rainbow trout, *Oncorhynchus mykiss* (Walbaum), J. Fish Dis. 28 (2010) 271–277.
[41] S.F. Snieszko, A symposium on diseases of fishes and shellfishes, Trans. Am. Microsc. Soc. 90 (1970) 190–208.
[42] A. Decostere, F. Haesebrouck, L.A. Devriese, Characterization of four *Flavobacterium columnare* (*Flexibacter columnaris*) strains isolated from tropical fish, Vet. Microbiol. 62 (1998) 35–45.
[43] J. Xu, X. Zhang, Y. Luo, X. Wan, Y. Yao, L. Zhang, Y. Yu, T. Ai, Q. Wang, Z. Xu, IgM and IgD heavy chains of yellow catfish (*Pelteobagrus fulvidraco*): molecular cloning, characterization and expression analysis in response to bacterial infection, Fish Shellfish Immunol. 84 (2019) 233–243.
[44] Y. Luo, W. Yu, Y. Yu, S. Dong, Y. Yin, Z. Huang, X. Wan, L. Zhang, Y. Yu, T. Ai, Q. Wang, Z. Xu, Molecular characterization and expression analysis of T cell receptor (TCR) γ and δ genes in dojo loach (*Misgurnus anguillicaudatus*) in response to bacterial, parasitic and fungal challenge, Fish Shellfish Immunol. 86 (2019) 641–652.
[45] T. Osamu, A. Shizuo, Pattern recognition receptors and inflammation, Cell 140 (2010) 805–820.
[46] F. Geissmann, M.G. Manz, S. Jung, M.H. Sieweke, M. Merad, K. Ley, Development of monocytes, macrophages, and dendritic cells, Science 327 (2010) 656–661.
[47] S.P. Ballou, G. Lozanski, Induction of inflammatory cytokine release from cultured human monocytes by C-reactive protein, Cytokine 4 (1992) 361–368.
[48] W. Zhou, Y. Zhang, Y. Wen, W. Ji, Y. Zhou, Y. Ji, X. Liu, W. Wang, M. Asim, X. Liang, Analysis of the transcriptomic profilings of Mandarin fish (*Siniperca chuatsi*) infected with *Flavobacterium columnare* with an emphasis on immune responses, Fish Shellfish Immunol. 43 (2015) 111–119.
[49] L. Li, M.X. Chang, P. Nie, Molecular cloning, promoter analysis and induced expression of the complement component C9 gene in the grass carp *Ctenopharyngodon idella*, Vet. Immunol. Immunopathol. 118 (2007) 270–282.
[50] X. Chen, W. Kong, Y. Yu, S. Dong, Z. Huang, W. Yu, J. Xu, Y. Luo, Q. Wang, Z. Xu, Molecular characterization and expression analysis of interleukin 15 (IL15) and interleukin-15 receptor subunit alpha (IL15Rα) in dojo loach (*Misgurnus anguillicaudatus*): their salient roles during bacterial, parasitic and fungal infection, Mol. Immunol. 103 (2018) 293–305.
[51] F. Sun, E. Peatman, C. Li, S. Liu, Y. Jiang, Z. Zhou, Z. Liu, Transcriptomic signatures of attachment, NF-κB suppression and IFN stimulation in the catfish gill following columnaris bacterial infection, Dev. Comp. Immunol. 38 (2012) 169–180.
[52] S. Li, Q. Guo, S. Li, H. Zheng, S. Chi, Z. Xu, Q. Wang, Glutamine protects against LPS-induced inflammation via adjusted NODs signaling and enhanced immunoglobulins secretion in rainbow trout leukocytes, Dev. Comp. Immunol. 98 (2019) 148–156.
[53] X. Zhen, P. David, G. Daniela, S. Irene, Z. Yong-An, V.G.J.R. Louise, H. Rasmus Demuth, B. Kurt, L.P. Scott, S. J Oriol, Teleost skin, an ancient mucosal surface that elicits gut-like immune responses, Proc. Natl. Acad. Sci. U.S.A. 110 (2013) 13097–13102.
[54] L. Zhang, Z. Liu, J. Fu, Q. Zhang, H. Bei, P. Nie, Immunogenicity and protective role of antigenic regions from five outer membrane proteins of *Flavobacterium columnare* in grass carp *Ctenopharyngodon idella*, Chin. J. Oceanol. Limnol. 34 (2016) 1247–1257.
[55] Y.A. Zhang, I.J. Salinas, D. Parra, S. Bjork, Z. Xu, S.E. Lapatra, J. Bartholomew, J.O. Sunyer, IgT, a primitive immunoglobulin class specialized in mucosal immunity, Nat. Immunol. 11 (2010) 827–835.
[56] Z. Xu, F. Takizawa, D. Parra, D. Gómez, G.J.L. Von, S.E. Lapatra, J.O. Sunyer, Mucosal immunoglobulins at respiratory surfaces mark an ancient association that predates the emergence of tetrapods, Nat. Commun. 7 (2016) 10728.
[57] J.C. Rathmell, Metabolism and autophagy in the immune system: immunometabolism comes of age, Immunol. Rev. 249 (2012) 5–13.
[58] T.G. Naidu, F.H. Newbould, Glycogen in leukocytes from bovine blood and milk, Can. J. Comp. Med. 37 (1973) 47–55.
[59] K.R. Feingold, I. Staprans, R.A. Memon, A.H. Moser, J.K. Shi- genaga, W. Doerrler, C.A. Dinarello, C. Grunfeld, Endotoxin rapidly induces changes in lipid metabolism that produce hypertriglyceridemia: low doses stimulate hepatic tri- glyceride production while high doses inhibit clearance, J. Lipid Res. 33 (1992) 1765–1776.
[60] G.H. Chen, Z. Luo, F. Chen, X. Shi, Y.F. Song, W.J. You, X. Liu, PPAR alpha, PPAR gamma and SREBP-1 pathways mediated waterborne iron (Fe)-induced reduction in hepatic lipid deposition of javelin goby *Synechogobius hasta*, Comp. Biochem. Physiol. C Toxicol. Pharmacol. 197 (2017) 8–18.
[61] S.K. Bose, S. Shrivastava, K. Meyer, R.B. Ray, Hepatitis C virus activates the mTOR/S6K1 signaling pathway in inhibiting IRS-1 function for insulin resistance, J. Virol. 86 (2012) 6315–6322.
[62] S. Zurab, R.T. Sterk, S.A. Deharo, M.A. Ozbun, Cellular entry of human papillomavirus type 16 involves activation of the phosphatidylinositol 3-kinase/Akt/mTOR pathway and inhibition of autophagy, J. Virol. 87 (2013) 2508–2517.
[63] Y.B. Wang, L.M. Weiss, A. Orlofsky, Intracellular parasitism with Toxoplasma gondii stimulates mammalian-target-of-rapamycin-dependent host cell growth despite impaired signalling to S6K1 and 4E-BP1, Cell Microbiol. 11 (2010) 983–1000.
[64] I. Tattoli, M.T. Sorbara, D. Vuckovic, A. Ling, F. Soares, L.M. Carneiro, C. Yang, A. Emili, D. Philpott, S. Girardin, Amino acid starvation induced by invasive bacterial pathogens triggers an innate host defense program, Cell Host Microbe 11 (2012) 563–575.
[65] I. Tattoli, D.J. Philpott, S.E. Girardin, The bacterial and cellular determinants controlling the recruitment of mTOR to the Salmonella-containing vacuole, Biol. Open 1 (2012) 1215–1225.
[66] K. Anne Marie, A.R. Woolery, O. Kim, Manipulation of kinase signaling by bacterial pathogens, JCB (J. Cell Biol.) 195 (2011) 1083–1092.
[67] C.J. Tai, S.J. Chang, P.C. Leung, C.R. Tzeng, ATP activates nuclear translocation of mitogen-activated protein kinases in human granulosa-luteal cells, Nat. Rev. Immunol. 13 (2013) 679–692.
[68] R. Anan, B. Tina, G. Viola, S. Kerstin, B. Anna-Lisa, B. Michael, Drosophila Ras/MAPK signalling regulates innate immune responses in immune and intestinal stem cells, EMBO J. 30 (2014) 1123–1136.
[69] K. Orth, L.E. Palmer, Z.Q. Bao, S. Stewart, A.E. Rudolph, J.B. Bliska, J.E. Dixon, Inhibition of the mitogen-activated protein kinase kinase superfamily by a Yersinia effector, Science 285 (1999) 1920–1923.
[70] V. Reiterer, L. Grossniklaus, T. Tschon, C.A. Kasper, I. Sorg, C. Arrieumerlou, *Shigella flexneri* type III secreted effector OspF reveals new crosstalks of proinflammatory signaling pathways during bacterial infection, Cell. Signal. 23 (2011) 1188–1196.
[71] H. Masanori, T. Masato, N. Takashi, H. Hideki, T. Kohsuke, M. Masao, W. Kenta, S. Takashi, W. Masahisa, Inactivation of the MAPK signaling pathway by *Listeria monocytogenes* infection promotes trophoblast giant cell death, Front. Microbiol. 6 (2015) 1145.