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Functional identification and expressional responses of large yellow croaker (*Larimichthys crocea*) interleukin-8 and its receptor

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ABSTRACT

Interleukin-8 (IL-8 or chemokine (C-X-C motif) ligand 8, CXCL8) is a chemokine produced by multiple cell types. It promotes chemotaxis and phagocytosis via interaction with chemokine receptors CXCR1 and CXCR2. Using published data, IL-8 gene (*LcIL-8*) of the large yellow croaker (*Larimichthys crocea*) was cloned into the pcDNA3.1 plasmid, and an interleukin-8 receptor (*LcCXCR2*) was cloned into the pEGFP-N1 plasmid. Secratory expression of *Lc*IL-8 in HEK293T cells was carried out, and product in culture medium was collected for *Lc*CXCR2 stimulation in HEK293 cells. Following receptor internalization observation and intracellular signaling detection, the functional interaction of *Lc*IL-8 and *Lc*CXCR2 was further determined and the ERK phosphorylation signal activation mediated by *Lc*CXCR2 was demonstrated. Quantitative real-time PCR analysis was used to analyze transcription level regulation of *Lc*IL-8 and *Lc*CXCR2 in various tissues of large yellow croaker. Expression of *LcIL-8 and LcCXCR2* was elevated in the spleen, head kidney, and liver after *Vibrio parahemolyticus* challenge. Results illustrated the functional interaction between *Lc*IL-8 and *Lc*CXCR2 in mediating intracellular ERK1/2 phosphorylation signaling and suggested that the *Lc*IL-8 and *Lc*CXCR2 system is part of the immune response induced by *V. Parahemolyticus* in *L. crocea*.

1. Introduction

Interleukins (ILs) are a group of cytokines first reported in secretions of white blood cells (leukocytes). The name, interleukins, was introduced in 1979 to replace various other terms previously used [1,2]. Different families of interleukins play important roles in diverse biological processes, especially immune responses, including interleukin-8 (a chemokine with C-X-C motif, named IL-8 or CXCL8) [3–5], one such cytokine. In humans, IL-8 protein is encoded by the *CXCL8* gene [6] and is initially produced as a precursor peptide with 99 amino acids. This form is cleaved into several active IL-8 isoforms with funtional interaction activity to its receptors [7,8]. IL-8 production is observed in a wide variety of species with similar tissue distribution and biological function, although variability is noted in lengths of precursor proteins and amino acid sequences. IL-8 functions include initiating chemotaxis in target cells toward the sites of infection, phagocytosis and angiogenesis by intracellular Ca^{2+}, exocytosis (e.g., histamine release), and respiratory burst initiation [9,10]. IL-8 signals through two G protein-coupled receptors CXCR1 and CXCR2 (also known as IL-8RA and IL-8RB in humans). These receptors are normally located on the surface of leukocytes and endothelial cells [11]. CXCR1 and CXCR2 are “shared” receptors that can be activated by IL-8 or other chemokines, thus mediating multiple intracellular signaling pathways, such as activation of phospatidylinositol-3-kinase (PI3K), mitogen-activated protein kinase (MAPK), phospholipase c (PLC), non-receptor tyrosine kinases, and Rho-GTPases [5,9,12]. The functional role of IL-8/CXCR1/2 signaling system has been well studied in human immune response, and its crucial role in inflammation-driven immunoregulatory processes following pathogenic invasion cancer identified [13,14].

In fishes, the immunoregulatory function of this signaling pathway has also been investigated and multiple *CXCL8* and *CXCR1/2* genes have been identified and characterized from various species. For example, homologous *CXCL8* genes were cloned from grass carp (*Ctenopharyngodon idellus*) [15], ayu (*Plecoglossus altivelis*) [16], zebrafish (*Danio rerio*) [17], rainbow trout (*Oncorhynchus mykiss*) [18,19], banded dogfish (*Triakis scyllia*) [20], black sea bream (*Acanthopagrus*

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schlegeli) [21], half-smooth tongue sole (*Cynoglossus semilaevis*) [22] and large yellow croaker (*Larimichthys crocea*) [23]. Phylogenetic analysis of these genes suggests three subgroups of *CXCL8* homologues in fishes and provides evidence for regulatory activity of IL-8 in immune responses of fish species.

In addition, IL-8 receptors, CXCR1 and CXCR2 were also identified from a wide range of fish species following their initial discovery in peritoneal leucocytes of carp (*Cyprinus carpio*) [24], including rainbow trout (*Oncorhynchus mykiss*) [25,26], zebrafish (*Danio rerio*) [5,27], and large yellow croaker (*L. crocea*) [28]. A substantial amount of bioinformatic and experimental data concerning the IL-8/CXCR1/2 system in fishes has been collected, which allows examination of functional interactions between the ligand IL-8 and signaling details of this system.

The large yellow croaker *L. crocea* is a commercially cultured marine fish in eastern and southern China. With the rapid development of its culture industry, infectious diseases are causing significant economic losses mainly due to various marine pathogens [29]. Based on published data for *CXCL-8* and *CXCR2* gene and functional activities [23,28], molecular properties of CXCR2 are further described to provide direct evidence on the interaction of IL-8 and CXCR2, and to analyze transcriptional responses of this signaling system in large yellow croaker following bacterial challenge. Results identify membrane localization of *Lc*CXCR2, internalization of *Lc*CXCR2 activated by *Lc*IL-8, *Lc*IL-8-mediated ERK1/2 phosphorylation activation in *Lc*CXCR2-expressing HEK293 cells, and suggest that the IL-8/CXCR2 system is involved in immunoregulatory processes in large yellow croaker after *Vibrio Parahemolyticus* infection. Results thus provide a deeper understanding of the IL-8/CXCR1/2 signaling pathway and its immunoregulatory role in fish.

2. Materials and methods

2.1. Challenge experiments and cDNA preparation

Healthy large yellow croaker (weight 150.4 ± 23.1 g) were purchased from a mari-culture farm in Zhoushan, Zhejiang Province, China. Fish were maintained at 25 °C in aerated seawater (salinity range: 31.08–32.17, refreshed daily) for 10 days prior to beginning the challenge experiment. After acclimation, two groups of 50 fish each were injected intramuscularly with sterilized phosphate buffered saline (PBS, pH 7.4) at a dose of 0.3 mL/100 g fish as a control, or with *V. Parahemolyticus* (1×10^8 CFU/mL, resuspended in PBS, pH 7.4). The gill, liver, head kidney, spleen, heart and muscle tissues were dissected from six healthy fish per group, and liver, head kidney and spleen were collected at 0, 6, 12, 24, 48, 72 and 96 h after injection. Tissue samples were frozen and stored immediately in liquid nitrogen for further analysis.

Total RNA was isolated from tissue samples using TRIzol reagent (TaKaRa, Kusatsu, Japan) and phenol chloroform. Integrity of total RNA was verified by electrophoresis, and RNA concentration and quality were determined using a Nanodrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA). For each sample, 1 μg total RNA was reverse transcribed into single-stranded cDNA via incubation with M-MLV reverse transcriptase and oligo(dT)20 (Promega Inc., Shanghai, China) at 42 °C for 1 h. An RNase inhibitor (Promega Inc., Shanghai, China) was used during cDNA synthesis. cDNA was kept at −20 °C for further assays.

2.2. Sequences characterization and the mammalian expression vectors construction

The coding sequence of *LcCXCR2* was cloned using forward primer (*Lc*CXCR2-seq-F) and reverse primer (*Lc*CXCR2-seq-R) (Supplementary Table 1) and further sequenced to confirm correction of CDS. The amino acid sequence was predicted with DNAMAN 8.0 and physicochemical properties projected with Protparam (http://www.expasy.org/tools/protparam.html). Analysis of secondary structure was predicted with PredictProtein (http://www.predictprotein.org/). *Lc*CXCR2 protein structure was predicted using SWISS-MODEL (http://swissmodel.expasy.org/).

To construct the *Lc*CXCR2 plasmid, reverse transcript PCR (RT-PCR) was used as described in "cDNA preparation". To amplify CDS of *LcCXCR2*, forward primer (*Lc*CXCR2-vec-F) and reverse primers (*Lc*CXCR2-vec-R-EGFP and *Lc*CXCR2-vec-R-Flag) were designed based on the *LcCXCR2* sequence (GenBank accession number: MK098550) to allow for subcloning into the pEGFP-N1 and pCMV-Flag plasmids, respectively (Supplementary Table 1). pEGFP-N1 and pCMV-Flag vectors were purchased from Clontech Laboratories, Inc. (Palo Alto, CA), and Sigma (St.Louis, MO). PCR products were inserted into the final pEGFP-N1 and pCMV-Flag expression vectors using the EcoR I and KpnI restriction enzymes (Beyotime, Shanghai, China) and Rapid DNA Ligation Kits (Beyotime, Shanghai, China). Constructed vectors were named *Lc*CXCR2-EGFP and Flag-*Lc*CXCR2, respectively, and sequenced to verify sequence fidelity, orientation, and reading frame.

The DNA sequence that includes the core coding region of the *Lc*IL-8 gene [23] (GenBank accession number: KP202400) with upstream KOZAK and signal peptide sequences (from pFLAG-CMV-3) was artificially synthesized (Supplementary Fig. 1) and subcloned into the pcDNA3.1 plasmid (Wuhan Transduction Bio, Wuhan, China). Constructed vectors were named SEC-*Lc*IL-8 and were sequenced to verify sequence fidelity, orientation, and reading frame.

2.3. Cell culture and transfection

Human embryonic kidney cell lines (HEK293 and HEK293T) were maintained in Dulbecco's modified Eagle's medium (DMEM, Hyclone, Logan, UT, USA) supplemented with 10% FBS, 100 U/mL penicillin, 100 mg/mL streptomycin and 4 mM L-glutamine (Thermo Fisher Scientific, Waltham, MA, USA) at 37 °C in a humidified incubator containing 5% CO2. *Lc*CXCR2-EGFP and Flag-*Lc*CXCR2 vectors were transfected into HEK293, and the SEC-*Lc*IL-8 plasmid construct was transfected into HEK293T cells by using X-tremeGENE HP (Roche), according to the manufacturer's instructions. Two days after transfection, stably expressing cells were selected by the addition of 800 mg/L G418.

2.4. Recombinant expression of LcIL-8 and LcCXCR2

HEK293T cells with or without expression of SEC-*Lc*IL-8 or empty vector (pcDNA3.1) were seeded into 10 cm cell culture dishes in DMEM (with 10% FBS, 100 U/mL penicillin, 100 mg/mL streptomycin and 4 mM L-glutamine). Twenty-four hours later, culture medium was replaced by fresh DMEM (with 10% FBS and 4 mM L-glutamine) and then collected after 4, 8 and 12 h.

HEK293 cells expressing *Lc*CXCR2-EGFP were seeded onto glass coverslips coated with 0.1 mg/ml of poly-L-lysine and allowed to attach overnight under normal growth conditions. After 24 h, cells were starved for a further 2 h in serum-free medium to eliminate the effects of FBS.

2.5. Receptor localization and translocation assay by confocal microscopy

For receptor surface expression analysis, cells were stained with the membrane probe DiI (Beyotime, Shanghai, China) at 37 °C for 5–10 min, fixed with 4% paraformaldehyde for 15 min, and finally incubated with DAPI (Beyotime, Shanghai, China) for 10 min. Cells were then visualized by fluorescence microscopy on a Leica TCS SP5II laser scanning confocal microscope using a HCX PL APO lambda blue 63 × 1.4 oil immersion len.

For receptor translocation detection, HEK293T cells with or without expression of SEC-*Lc*IL-8 or empty vector (pcDNA3.1) were seeded into 10 cm cell culture dishes in DMEM (with 10% FBS, 100 U/mL

penicillin, 100 mg/mL streptomycin and 4 mM L-glutamine). Twenty-four hours later, culture medium was replaced by fresh DMEM (with 10% FBS and 4 mM L-glutamine) and medium collected after 8 h. Medium was diluted 1/100 with DMEM (without FBS) and used to replace the medium of cells expressing *Lc*CXCR2-EGFP. After 0, 5, 15, 30 and 60 min at 37 °C, cells were fixed with 4% paraformaldehyde for 15 min. Cells were then visualized by fluorescence microscopy on a Leica TCS SP5II laser scanning confocal microscope using a HCX PL APO lambda blue 63 × 1.4 oil immersion len.

2.6. Western blot assay

To examine phosphorylation of ERK, cells that expressed *Lc*CXCR2 were incubated for 5 min with different culture media (with or without secretory expressed *Lc*IL-8) [30]. Subsequently, cells were lysed at 4 °C for 30 min with lysis buffer (Beyotime, Shanghai, China) that contained a protease inhibitor (Roche) on a rocker and then scraped. Proteins were then electrophoresed on a 10% SDS polyacrylamide gel and transferred to PVDF membranes. Membranes were blocked with 5% skim milk, then probed with rabbit anti-phospho-ERK1/2(Thr^{202}/Tyr^{204}) antibody (1:2000; Cell Signaling Technology), followed by detection by using HRP-conjugated goat anti-rabbit IgG (Beyotime, Shanghai, China). Blots were stripped and reprobed by using anti-ERK1/2 antibody (1:2000; Cell Signaling Technology) as a control for protein loading.

Immunoreactive bands were detected with an enhanced chemiluminescent substrate (Beyotime, Shanghai, China), and the membrane scanned using a Tanon 5200 Chemiluminescent Imaging System (Tanon Science & Technology, Shanghai, China). Intensities of bands were quantified by using the Bio-Rad Quantity One imaging system.

2.7. Real-time quantitative PCR (qRT-PCR)

cDNA samples from various tissues at different stages of challenge were used for qRT-PCR analysis. *β-actin* (ACTB) was amplified as an internal control (housekeeping) gene, and gene-specific primers for *β-actin* and *LcIL-8* were synthesized following published methods [23]. Specific qRT-PCR primers for *LcCXCR2* were designed based on the CDS region (Supplementary Table 1). Primers were tested to ensure amplification of single discrete bands with no primer dimers. qRT-PCR assays were carried out using SYBR PrimeScript™ RT reagent Kits (TaKaRa, Kusatsu, Japan) following the manufacturer's instructions, and ABI 7500 Software v2.0.6 (Applied Biosystems, UK). qRT-PCR was performed for 35 cycles under the following conditions: 95 °C/5 s, 60 °C/30 s. The relative level of gene expression was calculated using the $2^{-\triangle\triangle Ct}$ method and data were normalized by geometric averaging of internal control genes [31]. Differences between experimental and control groups were tested using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test, using PASW Statistics 18.00 (SPSS Inc., Chicago, IL, USA). Significance was set at $P < 0.05$, and extreme significance was set at $P < 0.01$.

3. Results

3.1. Characterization of L. crocea LcCXCR2

The CDS region of *LcCXCR2* cloned from *L. crocea* spleen (GenBank accession number: MK098550) was 1083 bp long encoding and encoded a protein with 360 amino acid residues. This protein was predicted to have a molecular mass of 40.56 kDa and an isoelectric point (pI) of 8.95. The three-dimensional (3D) structure of the *Lc*CXCR2 protein is shown in Fig. 1A and supplementary video, and its secondary structure in Fig. 1B. Homology modeling revealed similarity between this segment and the 2lnl.1.A segment from the Protein Data Bank. Fusion expression, enhanced green fluorescent protein (EGFP) tagged at the C-terminal, of *Lc*CXCR2 in HEK293 cells was used and localization of *Lc*CXCR2-EGFP was demonstrated by confocal microscopy on the plasma membrane as a transmembrane receptor.

Supplementary video related to this article can be found at https://doi.org/10.1016/j.fsi.2019.01.035.

3.2. LcIL-8 induced internalization of LcCXCR2-EGFP

The fusion expression of *Lc*CXCR2-EGFP in HEK293 cells was conducted an further stimulated by *Lc*IL-8 in culture medium. *Lc*CXCR2 internalization upon the activation of *Lc*IL-8 in HEK293 cells was detected, and dramatic redistribution of *Lc*CXCR2 in the cytoplasm with distinct perinuclear accumulation was observed (Fig. 2). Treatment of cells with 1.0% *Lc*IL-8 in culture medium evoked rapid internalization of *Lc*CXCR2-EGFP in HEK293. After exposure to *Lc*IL-8, internalized receptors became distributed throughout the cytoplasm. At 15 and 30 min, these receptors were largely clustered in the perinuclear region.

3.3. LcIL-8 mediated ERK1/2 phosphorylation

ERK1/2 activation was assessed after *Lc*IL-8 administration in HEK293 cells expressing *Lc*CXCR2. From Fig. 3, notable ERK1/2 phosphorylation activity mediated by *Lc*IL-8 is detected by western blot assay.

3.4. Transcriptional level of LcIL-8 and LcCXCR2 in different tissues of L. crocea

Quantitative real-time PCR was used to determine tissue distribution of *LcIL-8* and *LcCXCR2* gene expression in gill, liver, head kidney, spleen, heart and muscle of *L. crocea*. The gene expression levels in these tissues were expressed relative to those in gill. As shown in Fig. 4, *LcIL-8* was widely expressed in all six tissues tested with significantly higher expression levels in heart, muscle and liver. Meanwhile, *LcCXCR2* was also detected in all tissues but with a different expression profile (significantly higher expression in spleen, head kidney and liver).

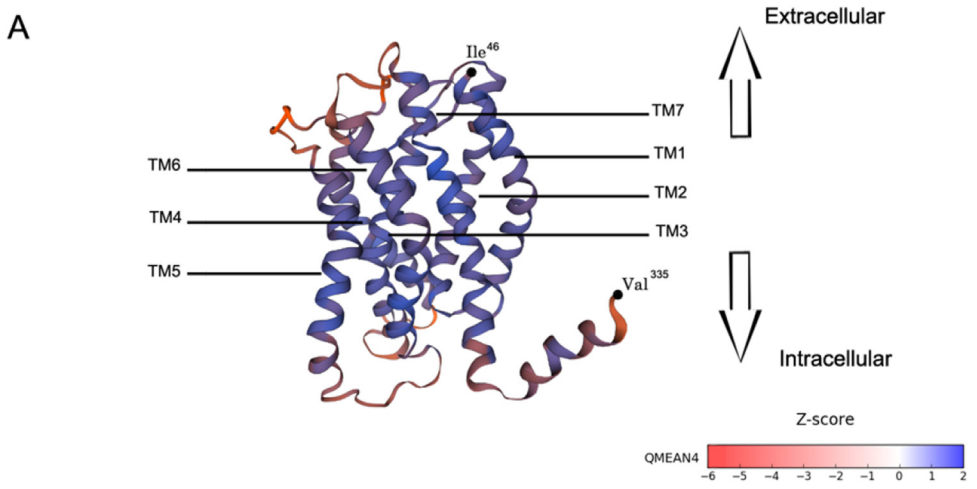
3.5. Transcriptional regulation of LcIL-8 and LcCXCR2 in spleen, head kidney and liver of L. crocea in response to V. Parahemolyticus challenge

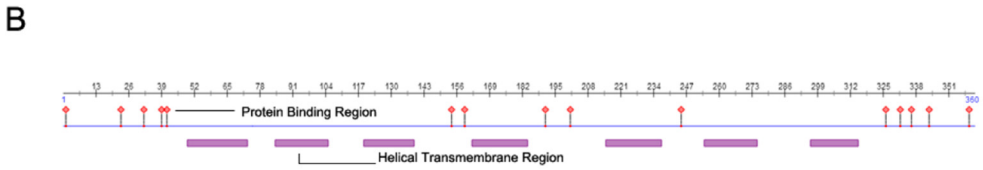
The expression level variations in spleen, head kidney and liver revealed during the bacterial challenge experiments are shown in Fig. 5. After *Vibrio Parahemolyticus* injection, a notable *LcIL-8* and *LcCXCR2* mRNA expression level increase was detected by the qPCR assays. Consistent transcriptional change profiles of *LcIL-8* in all three tissues are shown in Fig.5 Ã C. Peak values of relative gene expression of *LcIL-8* were found at 6 h after injection, with a highly significant increase detected in spleen (25.82 fold) and head kidney (28.68 fold) ($p < 0.001$) and significant up-regulated level in liver (3.91 fold). After 12 h post-injection, *LcIL-8* expression was reduced compared with expression at 6 h, and a significant reduction compared to control was observed at 72 h post-injection. At 96 h post-injection, *LcIL-8* expression had returned to a level similar to that of the control.

LcCXCR2 gene expression was also observed in all three tissues (Fig. 5 D ~ F). In spleen, the transcriptional level of *LcCXCR2* was significantly increased at 12 h post-injection followed by a peak value at 24 h (48.12 fold), then a decrease from 48 h (compared the peak at 24 h) to a significant reduction at 96 h (compared to control). Similar expression profiles of *LcCXCR2* were detected in head kidney but with peak value at earlier time (12 h). In liver, the variation of *LcCXCR2* expression is also stable with same profile in head kidney.

4. Discussion

IL-8 is a well-known cytokine that plays crucial roles in inflammatory responses and is activated in response to various diseases





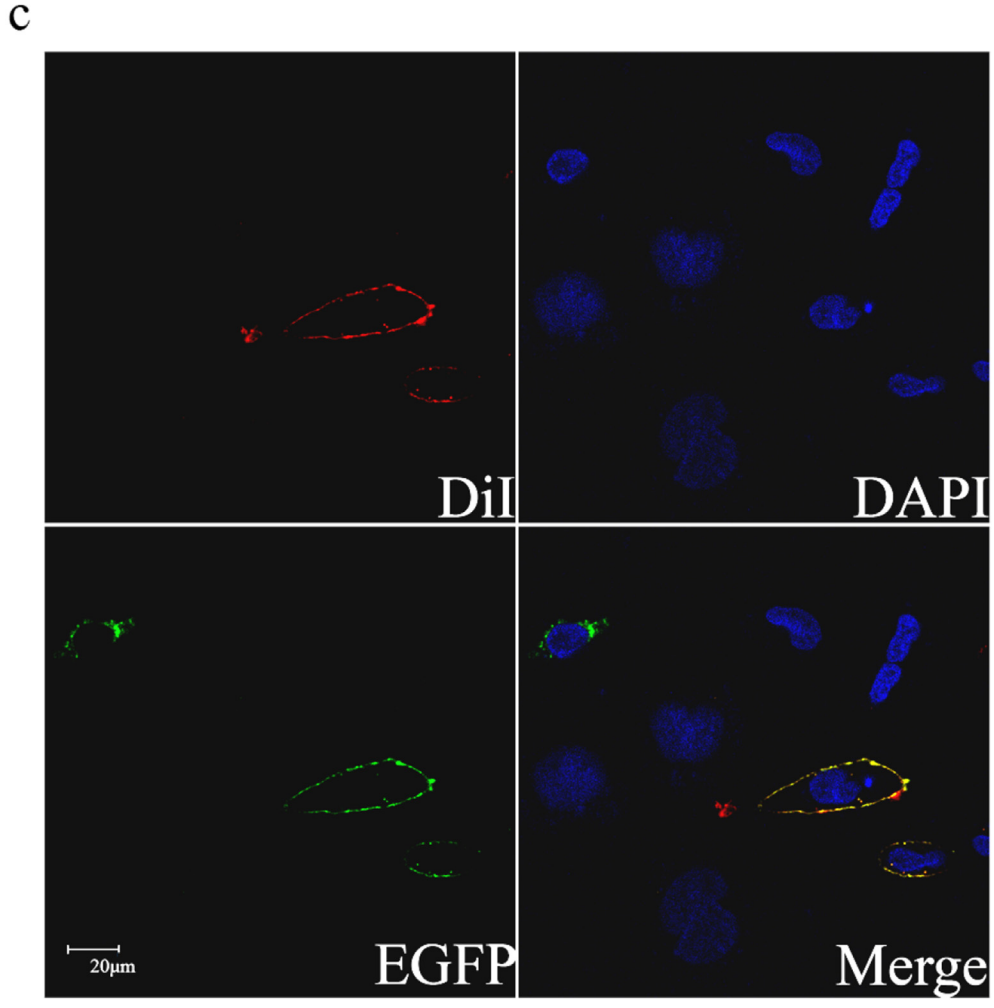


Fig. 1. Predicted *Lc*CXCR2 protein structure, respective domain and subcellular location of *Lc*CXCR2-EGFP. (A) Predicted 3D structure of the *Lc*CXCR2 protein. Protein-binding regions were represented by black dots. Seven transmembrane domains (TM1–TM7). The predicted 3D structure of the *Lc*CXCR2 protein was generated using SWISS-model. (B) *Lc*CXCR2 protein binding domain and transmembrane region. The dashboard overview was generated using PredictProtein. (C) *Lc*CXCR2-EGFP expressing cells were stained with cell membrane probe (DiI) and cell nucleus probe (DAPI) and detected by confocal microscopy. All images are representative of at least three independent experiments.

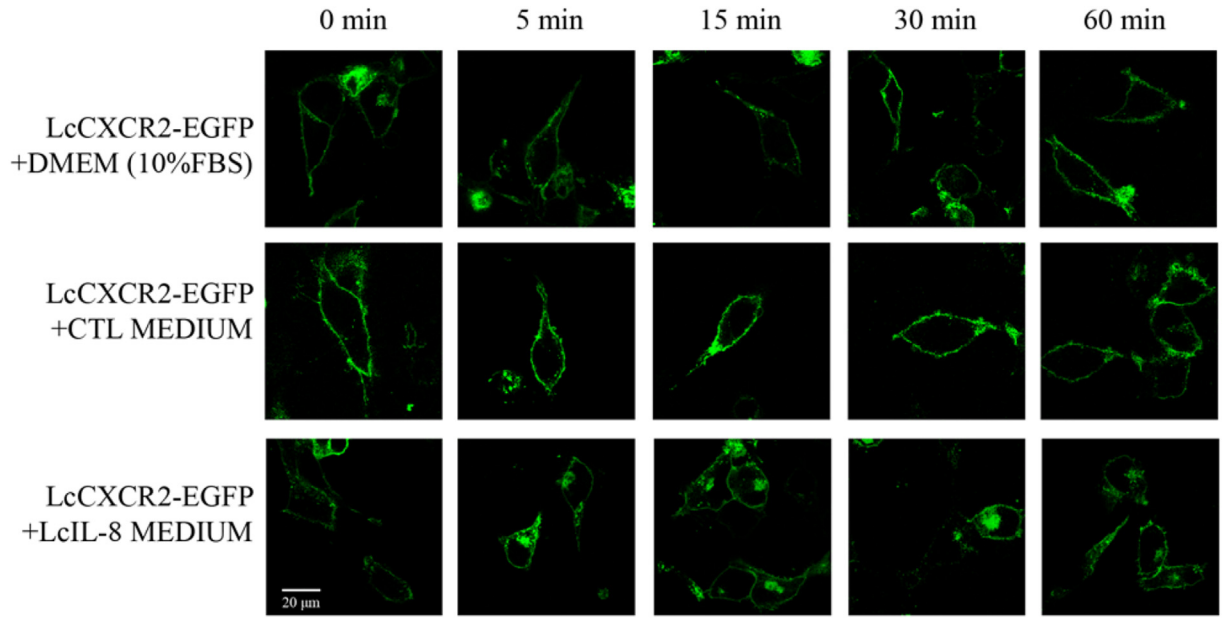


Fig. 2. Internalization of overexpressed *Lc*CXCR2 initiated by 1/100 diluted *Lc*IL-8 in cell culture medium in stable *Lc*CXCR2-EGFP expressing HEK293 cells determined by confocal microscopy. CTL MEDIUM and *Lc*IL-8 MEDIUM indicates the *Lc*CXCR2-EGFP expressing HEK293 cells is incubated with the 1% cell culture medium collected from pcDNA3.1 empty vector and *Lc*IL-8 expressing HEK293T cells respectively.

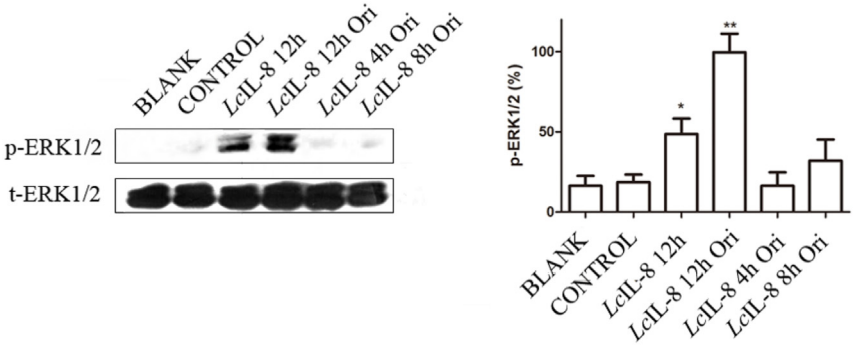


Fig. 3. Activation of ERK1/2 in *Lc*CXCR2 expressed HEK293 in response to *Lc*IL-8. BLANK indicates *Lc*CXCR2 expressed HEK293 treated by DMEM with 1% cell culture medium collected from HEK293T cells at 12 h, CONTROL indicates *Lc*CXCR2 expressed HEK293 treated by DMEM with 1% cell culture medium collected from pcDNA3.1 empty transfected HEK293T cells at 12 h, *Lc*IL-8 12 h indicates *Lc*CXCR2 expressed HEK293 treated by DMEM with 1% cell culture medium collected from SEC-*Lc*IL-8 transfected HEK293T cells at 12 h, *Lc*IL-8 12 h Qri indicates *Lc*CXCR2 expressed HEK293 treated by 100% cell culture medium collected from SEC-*Lc*IL-8 transfected HEK293T cells at 12 h, *Lc*IL-8 4 h Qri indicates *Lc*CXCR2 expressed HEK293 treated by 100% cell culture medium collected from SEC-*Lc*IL-8 transfected HEK293T cells at 4 h, *Lc*IL-8 8 h Qri indicates *Lc*CXCR2 expressed HEK293 treated by 100% cell culture medium collected from SEC-*Lc*IL-8 transfected HEK293T cells at 8 h. Error bars, S.E. for three independent experiments. One-way ANOVA with Tukey's post hoc test revealed differences from vehicle (0 min) (*, $p < 0.05$; **, $p < 0.01$). All experiments were repeated independently at least three times with similar results.

[32–34]. Its cognate G protein-coupled receptors, CXCR1 and CXCR2, are closely related and can specifically recognize IL-8 and begin a series of intracellular signaling events to regulate cell functions [35]. In the present study, the full lengths of *IL-8* and *CXCR2* CDS regions from the spleen of large yellow croaker, which had been previously identified and characterized, were cloned [23,28]; the coding sequence of *Lc*IL-8 mature peptide (23–99 aa) was subcloned into pcDNA3.1 plasmid and expressed in HEK293T cells to produce functionally active *Lc*IL-8 peptide, and the whole coding sequence of *Lc*CXCR2 (360 aa) was subcloned into pEGFP-N1 and pCMV-Flag vectors and expressed in HEK293 cells to examine the interaction between *Lc*IL-8 and *Lc*CXCR2. Secreted *Lc*IL-8 was used to stimulate *Lc*CXCR2-expressing HEK293 cells and subsequent receptor internalization and intracellular signaling detected by confocal microscopy and ERK1/2 activation assays. The physiological function of IL-8/CXCR2 system was further investigated in large yellow croaker following pathogen challenge with *V. parahemolyticus* [36].

CXCR2, also known as interleukin 8 receptor β, is a member of the G protein-coupled receptor family members (GPCRs) that bind to IL-8 [37]. In this study, the full length of CDS (Supplementary Fig. 1) was cloned, which completed a 147 bp fragment to 5' terminal of the published sequence [28], and the deduced amino acid sequence is 360 aa long. Using its homolog receptor, CXCR1 2lnl.1.A segment from the Protein Data Bank as a template, *Lc*CXCR2 topology was predicted by SWISS-MODEL protein structure homology-modeling server. As shown in Fig. 1A, the predicted *Lc*CXCR2 3D structure shows the typical seven-transmembrane domain (TM1-7) of GPCRs [38]. The structure is consistent with the secondary structure predicted as shown in Fig. 1B, and also reveals seven transmembrane domains. Furthermore, protein-binding regions were analyzed and showed ligand-binding sites on extracellular domains or G protein coupling domains in intracellular place. To determine the subcellular localization of *Lc*CXCR2, confocal microscopy was applied via fusion expression of *Lc*CXCR2-EGFP in HEK293 cells and significant cell surface expression of *Lc*CXCR2 was demonstrated (shown in Fig. 1C), suggesting that the C-terminal EGFP tag does not affect *Lc*CXCR2 orientation.

CXCR2 binds the agonist IL-8, thus mediating cellular responses. These responses are regulated by modifications on the receptor itself, leading to uncoupling of the receptor from G protein and desensitization of the receptor [39]. Two mechanisms of receptor phosphorylation by protein kinase C (PKC)-dependent and G protein-coupled receptor kinase (GRK)-dependent pathways are involved in CXCR2 down-

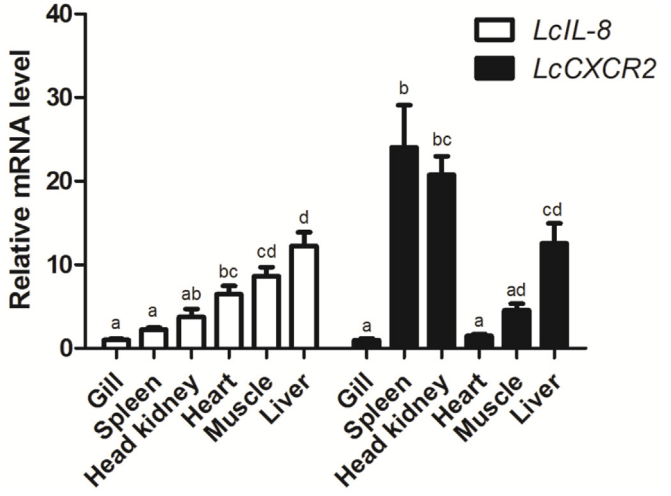


Fig. 4. Relative expression of *LcIL-8* and *LcCXCR2* mRNA in different tissues of *L. crocea*. Expression was normalized to expression of the internal control gene (*β-actin*) and to gill. Each value represents the mean ± SEM of n = 6 per group. Different lowercase letters above the bars indicate significant differences ($P < 0.05$) in different tissues.

regulation [40,41]. Agonist-induced internalization mediated by GRKs activation is a well-characterized phenomenon for GPCR responses to stimuli [42,43]. Mature *Lc*IL-8 peptide was expressed, based on published functional characterization data [23], in HEK293T cells, and *Lc*CXCR2 internalization response to *Lc*IL-8 administration was tested. Results showed that *Lc*CXCR2-EGFP fluorescence was dramatically and rapidly internalized in a time-dependent manner into the cytoplasm in response to addition of *Lc*IL-8-containing cell culture medium to HEK293 cells (Fig. 2). This result indicates that secreted *Lc*IL-8 is functionally active in HEK293T cells and in an interaction between *Lc*CXCR2 and *Lc*IL-8.

Following stimulation of CXCR2s by its ligand, intracellular signaling, such as ERK1/2 phosphorylation, is activated and further regulates downstream gene expression in target cells [44–46]. In the present study, ERK1/2 phosphorylation activation in Flag-*Lc*CXCR2-expressing HEK293 cells was tested after administration of secreted *Lc*IL-8 (Fig. 3). A significant increase in ERK1/2 phosphorylation was observed after stimulation of *Lc*IL-8-containing cell culture medium (collected at 12 h of secretion from HEK293T cells). These two experiments provide solid evidence of functional interaction between *Lc*IL-8 and *Lc*CXCR2, which is fundamental information to understanding the physical functions of this signaling system.

IL-8 and CXCR2 signaling is involved in multiple immunological responses. IL-8 engagement of CXCR2 on neutrophils results in the initiation and increase in migration of neutrophils distant from the site of inflammation, a process identified in zebrafish [17,47]. In previous immunological investigations of large yellow croaker after pathogen exposure, functional roles of CXCR2 and IL-8 were discussed and their involvement of inflammatory response to bacterial challenges demonstrated [23,28]. To better understand the systematic regulation of *Lc*IL-8 and *Lc*CXCR2 in large yellow croaker after bacterial challenge, transcriptional distribution of *LcIL-8* and *LcCXCR2* was measured simultaneously in various tissues after *V. Parahemolyticus* challenge. The widespread expression of *LcIL-8* in all tissues (Fig. 4), is mainly consistent with previously reports [23], except for higher expression in muscle which might be due to different physiological status. After pathogen

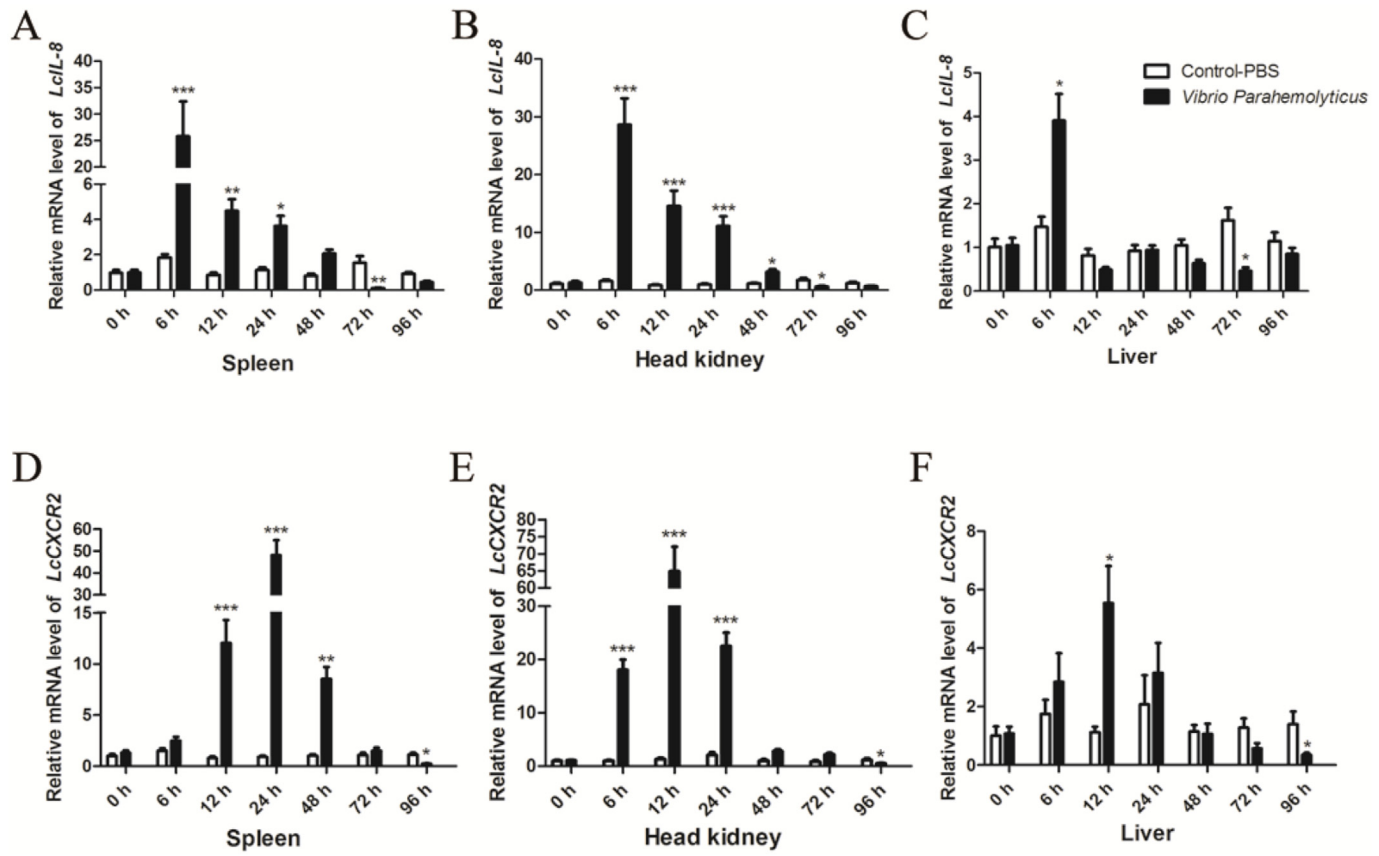


Fig. 5. Relative expression of *LcIL-8* and *LcCXCR2* mRNA in three tissues of *V. parahaemolyticus* challenged *L. crocea*. The expression was normalized to expression of the internal control gene (*β-actin*) and control group (PBS injected) at 0 h. Each value represents the mean ± SEM of n = 6 per group. Different lowercase letters above the bars indicate significant differences ($P < 0.05$) in different tissues.

injection, rapid upregulation of *LcIL-8* expression, followed by a gradual recovery, was observed in tissues of the spleen, head kidney and liver. This result is in agreement with previously published findings for CXCIL8-l1 in large yellow croaker [23] and CXCL8s in zebrafish [17], and strongly suggests that CXCIL8-l1 plays a role in acute inflammation. For *LcCXCR2*, predominant expression was detected in immune organs with a notably different distribution profile from *LcIL-8*, and a slower transcriptional adjustment, compared with expression of *LcIL-8*, is demonstrated with peak values of mRNA level at 24 h, 12 h and 12 h after bacterial infection in spleen, head kidney and liver respectively (Fig. 5) These results are mainly consistent with previous studies [28,48].

IL-8 is produced by a series of cell types including leukocytes such as monocytes and macrophages, as well as somatic cells such as endothelial cells or fibroblasts [49]; CXCR2 is mainly expressed in neutrocytes [50]. Notable differences in expression distribution profiles of *LcIL-8* and *LcCXCR2* in multiple tissues of large yellow croaker might be related with varying cell types in these organs. For instance, the high expression of *LcCXCR2* in immune organs (Fig. 4). It has been reported that expression of CXCR1 and CXCR2 can be down-regulated by activation of neutrophils by IL-8 or neutrophil-activating peptide [51,52]. However, our results, consistent with previous publications, demonstrated an elevation of *LcCXCR2* expression following the upregulation of *LcIL-8*. This may reflect cell migration to these immune organs [53].

In conclusion, *Lc*IL-8 (previously named as *Lyc*CXCL8_L1) was cloned and expressed in HEK293T cells, and the *Lc*CXCR2 receptor was identified and expressed in HEK293 cells. Functional interactions between *Lc*IL-8 and *Lc*CXCR2 were determined by observation of internalization and detection of ERK1/2 phosphorylation activity. The expression distribution and pathogen response changes in*LcIL-8* and *LcCXCR2* were analyzed and their immunoregulatory functions were defined.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.01.035.

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